The Role of Reactive Nitrogen Species and Aged Garlic Extract on Platelet Function

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Declaration

I declare that while registered as a candidate for the University's Research degree, I have not been a registered candidate or enrolled student for another award from LJMU or another academic institution

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Abstract

Natural therapies such as Aged Garlic Extract (AGE) have displayed cardioprotective properties, with studies indicating that AGE can inhibit platelet aggregation both *in vivo* and *in vitro*. The mechanism of inhibition induced by AGE is proposed to be due to AGE exerting effects upon several targets within platelets, including calcium and cyclic adenosine monophosphate (cAMP). The effect of AGE upon the other cyclic nucleotide, cyclic guanosine monophosphate (cGMP) is currently unknown. The aims therefore of this project are to identify the effect of AGE upon platelet cGMP, as well as associated signaling molecules including nitric oxide (NO) and cAMP.

It was found that the NO donor 3-morpholinosydnonimine (Sin-1) in high concentrations along with the presence of specific inhibitors inhibited platelet aggregation independently of cGMP. Experimentation using chemical inhibitors also displayed erratic results in the presence of high concentrations of AGE, indicating that AGE was influencing the binding of such inhibitors.

The results of *in vitro* experiments indicated that AGE moderately increases intraplatelet cGMP, whereas intraplatelet cAMP is significantly increased. it is proposed that the main mechanism of inhibition caused by AGE is due to increases in cAMP. As intraplatelet cAMP can also be influenced by intraplatelet cGMP, it is likely that cAMP is increased directly and indirectly by AGE.

Evidence provided in the present study supports the proposed theory that the mechanisms of inhibition of platelet aggregation by AGE is multimechanistic. More specifically inhibition of platelet aggregation by AGE is due to AGE increasing intraplatelet cyclic nucleotides, reducing the expression of key receptors such as GPIIb/IIIa and inhibiting agonist induced platelet shape change. As AGE can inhibit platelet aggregation, which is a key risk factor in cardiovascular disease, the consumption of AGE would be beneficial to those who are at risk of cardiovascular episodes.

Table of Contents

Contents

Chapter 1

Chapter 2

List of Figures

Chapter 1

Chapter 2

Chapter 3

List of Tables

Abbreviations

A_{2A}	Adenosine A_{2A} receptor
A_{2B}	Adenosine A_{2B} receptor
AA	Arachadonic acid
AC	Adenylyl / Adenylate cyclase
ADP	Adenosine diphosphate
AGE	Aged Garlic Extract
AGEPs	Advanced glycation end products
ANF	Atrial natriuretic peptide
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BH_4	Tetrahydrobiopterin
BSA	Bovine serum albumin
CaM	Calmodulin
cAMP	cyclic Adenosine Monophosphate
Ca^{2+}	Calcium
cGMP	cyclic Guanosine Monophosphate
COX	Cyclooxygenase
cNOS	Constituitive nitric oxide synthase
CVD	Cardiovascular disease
DAG	Diacylglycerol
$_dH_2O$	Distilled water
DAN	2,3-diaminoapthalene
DAPT	Dual anti-platelet therapy
DEA	Diethyl ether AGE extract
DMSO	Dimethyl sulfoxide
ECL	Enhance chemiluminescence
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EIA	Enzyme immunosorbent assay

eNOS / NOS3	Endothelial nitric oxide synthase
ERK	Extracellular regulated kinases
EtOH	Ethanol
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Guanylyl / Guanylate cyclase
GDP	Guanosine diphosphate
G-Proteins	Glycol proteins
GPIIb/IIIa	Fibrinogen receptor
GR 144053	4-[4-[4-(Aminoiminomethyl)phenyl]-1 -piperazinyl]-1-piperidineacetic acid trihydrochloride
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HEPES	Hexadimethyldisilazane
$HO^{\bullet}$	Hydroxyl Radical
HRP	Horseradish peroxidase
Hrs	Hours
IBMX	3-isobutyl-1-methylxanthine
IgG	Immunoglobulin G
iNOS / NOS2	Inducible nitric oxide synthase
IP_3	Phosphoinositol triphosphate
KCl	Potassium Chloride
JNK	c-Jun N-Terminal kinases
LDL	Low density lipid
MAPK	Mitogen activated protein kinases
MeOH	Methanol
MI	Myocardial infarction
Min	Minutes
$MgCl_2$	Magnesium Chloride
NaCl	Sodium Chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaF	Sodium fluoride
NaH_2PO_4	Sodium Phosphate

NaOH	Sodium hydroxide
NED	Naphtylethylene diaminedigydrochloride
NO_2	Nitrogen dioxide
NO_2^-	Nitrite anion
NO_3^-	Nitrate anion
NO	Nitric oxide
nNOS / NOS1	Neuronal nitric oxide synthase
O_2	Oxygen
$O_2^{-\bullet}$	Superoxide Anion
ODQ	1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxal in-1-one
$ONOO^-$	Peroxynitrite
$P2X_1$	Purigenic ADP receptor
$P2Y_1$	Purigenic ADP receptor
$P2Y_{12}$	Purigenic ADP receptor
p38	p38 Mitogen activated protein Kinase
PAF	Platelet activating factor
PAP-4D	Platelet aggregation profiler 4d
PAR1	Protease activated receptor 1
PAR4	Protese activated receptor 4
PBS	Phosphate buffered saline
PDGF	Platelet Derived Growth Factor
PDE	Phosphodiesterase
PG	Prostaglandin
pGC	Particulate guanylyl cyclase
$PGF_{1\alpha}$	Prostaglandin F_1
PGE_1	Prostaglandin E_1
PGI_2	Prostacyclin I_2
PIP_2	Phosphatidyl inositol biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
PLCβ	Phospholipase C beta

PMSF	Phenylmethylfulfonyl flouride
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PVDF	Polyvinylidene fluoride
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room Temperature
SAC	S-Allylcysteine
SEM	Standard error of the mean
SEMic	Scanning electron microscope
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sGC	Soluble guanylyl cyclase
Sin-1	3-Morpholinylsydnoneimine chloride
SNP	Sodium nitroprusside
SOCE	Store operated calcium entry
SOD	Superoxide dismutase
SQ 22536	9-(Tetrahydro-2-furanyl)-9*H*-purin-6 -amine
SYK	SRC kinases
TMB	3,3',5,5'-Tetramethylbenzidine
TN TBS	Tween Tris buffered saline
Tris	Tris (hydroxymethyl)aminomethane
TXA_2	Thomboxane A_2
TXB_2	Thromboxane B_2
VASP	Vasodilator-stimulated phosphoprotein
v/v	Volume to Volume
vWF	Von Willebrand factor
w/v	Weight to volume

Publications arising from work

Smith, S (2011) Aged Garlic Extract: A possible preventative therapy for thrombotic events, *JFST*, **25 (4)**, 26-27

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1 Introduction

Currently cardiovascular disease (CVD) has the highest mortality rate in the UK, accounting for 30 % of deaths, which surpasses the 28 % mortality rate of cancer (Cancer Research UK, 2010). In 2008, seventeen million people died from cardiovascular diseases (CVD) and with approximately 10 % of the global disease burden attributed to CVD, it remains the world's number one killer (World Health Organization 2011). In the UK alone, it is predicted that 1 in 3 of the population will die from vascular related diseases (Bhatnagar *et al* 2010). The pathogenesis of CVD is a complex process, which culminates in the appearance of arterial plaques, the consequences of which are restricted blood flow or plaque rupture, both of which may lead to the activation and aggregation of platelets, resulting in either myocardial infarction (MI) or stroke. Modern clinical treatment of these conditions has led to a high survival rate, for instance the mortality rates of stroke victims have halved over the past twenty years (Stroke Association 2013, Bhatnagar *et al* 2010). This decrease in the mortality rate is partially due to long-term pharmacological intervention for the treatment and prevention of recurrent stroke or MI (Stroke association 2013, Townsend *et al* 2012). One such treatment is statins, which reduce circulating cholesterol but are also reported to have pleiotropic properties (Mitsios *et al* 2010), which may improve the circulation and partially inhibit platelet activation. In conjunction with statins antiplatelet therapies are also administered, with the most common drug being aspirin, which inhibits thromboxane A_2 (TXA_2), a potent platelet activator. Other antiplatelet medications include clopidogrel that targets the ADP receptors and tirofiban, which target the platelet surface receptors GPIIb/IIIa. Recent scientific investigations of the physiology of platelets have determined intricate cell signalling mechanisms, that lead to shape change, expression of integrins and interaction of platelets with other cell types. The current therapies for platelets are quite successful but the increase in knowledge concerning platelet physiology allows a greater scope for novel anti-platelet therapies to be developed.

A number of natural products have been reported to have antiplatelet properties and therefore provide an area that is certainly worthy of further investigation. Several studies have indicated that natural products such as kiwi fruit, nettles and wines can

influence human platelets (O'Kennedy *et al* 2006, Duttaroy *et al* 2004, Pierre *et al* 2005, Wang *et al* 2002). For example an extract from tomato seeds (O'Kennedy *et al* 2006) was shown to have inhibitory effects on circulating human platelets. Natural products offer an array of different classes of phytochemicals, which may offer novel mechanisms to inhibit platelets. This thesis intends to demonstrate that an aged garlic extract (AGE), commercially termed Kyolic, can modulate human platelet activity. Prior to justifying and discussing research protocol, a review on platelet physiology and their biochemistry will be given, along with a detailed report on the properties and constituents of AGE.

1.1 Platelets in the vascular system

1.1.1 Production of platelets

Platelets are derived from megakaryotcytes, a precursor cell that is formed in the bone marrow, and were first described by Donné in 1842. Platelets are the second most populous blood cell after the red cell. The normal range for human circulating platelets is reported as 140-450 x 10^9 platelets/L with dimensions of 2–4 μm (Harrison 2005). Approximately one third of platelets are stored within the spleen, with the release of these excess stores into the circulation initiated by contraction of the spleen (Aster 1966). In those with normal platelet counts there remains a constant equilibrium between circulating platelets and those sequestered in the spleen (Wadenvik *et al* 1987, George and Rizvi 2001).

The formation of platelets is a process called thrombocytopoeisis, which involves the fragmentation of a megakaryocyte, it is these derived fragments that are known as platelets (Harrison 2005). Each megakaryocyte can produce 5000 - 10000 platelets, with a healthy adult producing 10^{11} platelets per day. The production of platelets is mediated by the hormone thrombopoeitin derived from the liver or kidneys (Batinelli *et al* 2001). The current model of platelet formation from megakarocytes indicates that mature megakarocytes extend long branches that are designated proplatelets, which are made up of platelet sized swellings connected by cytoplasmic bridges (Thon and Italiano 2010). The formation of a proplatelet is due to the erosion of one pole of the megakarocyte forming pseuopodial structures along with the rearrangement and polymerization of the

microtubules and actin structure (Hartwig and Italiano 2006). It is these proplatelets that are eventually fragmented away from the megakarocyte that derives a blood platelet (Malara and Balduini 2012).

Abnormal platelet count or functions disorders are known as thrombocytopathy which includes low platelet count thrombocytopenia or increased platelet count thrombocytosis (Brewer 2006). Dysfunctional platelets and activity are also associated with numerous other diseases such as diabetes (Stratmann and Tschoepe 2005), asthma (Kornerup and Page 2007) and CVD (Ricotta *et al* 2008).

1.1.2 Platelet physiology

Platelets are anucleate and possess a normal cell physiology with a cytosolic fraction, internal membrane system, cytoskeleton, mitochondria, lysosomes, and glycogen and storage granules (Hartwig and Italiano 2006). The storage granules consist of α granules and dense granules, both of which are important to platelet functions. α-granules store and secrete proteins such as fibrinogen, von Willebrand factor (vWF) and platelet derived growth factor, whereas dense granules contain Adenosine Diphosphate (ADP), Calcium (Ca^{2+}) and Adenosine Triphosphate (ATP) that help to initiate and support aggregation (Cimmino and Golino 2013) (Fig. 1.1).

A key factor in the coagulation cascade is thrombin, which is a potent activator of platelets (Brass 2003). Thrombin induces activation of the G-protein coupled protease activated receptors PAR1 and PAR4 on human platelets to initiate signalling cascades (Furie and Furie 2012). These cascades lead to increases in Ca^{2+}, secretion of autocrine activators, trafficking of adhesion molecules to the plasma membrane and shape change, all of which promote platelet aggregation (Austin *et al* 2013). The thrombin receptors are affected in a dose dependent manner, with PAR1 activated at low thrombin concentrations, whereas PAR4 is induced at higher thrombin concentrations (White 1999, Stalker *et al* 2012).

Platelets are host to numerous different receptors and receptor like proteins that regulate adhesion to glycoprotein receptors and integrins that help mediate platelet-to-platelet interaction or platelets to other cell types (Stalker *et al* 2012) (Fig 1.1).

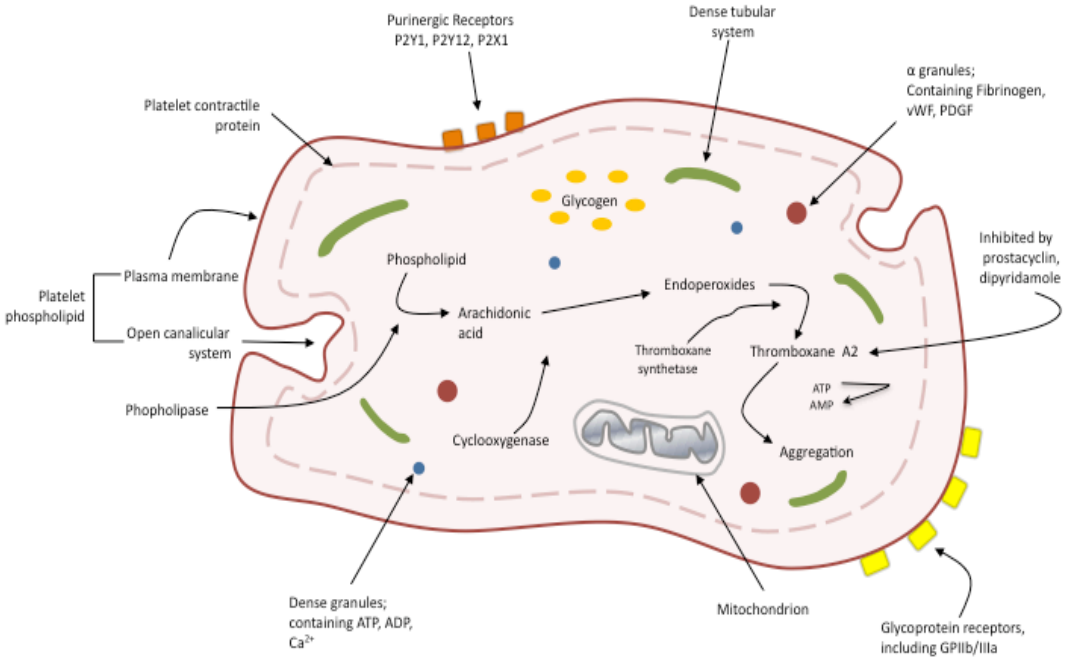


Fig. 1.1:- Diagram of a platelet. Dense granules contain molecules that help to sustain aggregation including ADP, Ca^{2+} and serotonin. α granules contain proteins such as fibrinogen and collagen as well as other clotting factors. Glycoproteins on the surface, including the collagen receptor Ia and the fibrinogen IIb/IIIa, are important in adhesion and aggregation. The plasma membrane and canalicular system provide a large reactive surface, on which plasma coagulation factors are absorbed and activated. Drugs such as aspirin inhibit platelet function by inhibiting cyclooxgenase whereas prostacyclins from endothelial cells and dipyridamole inhibit the action of thromboxane A_2.

1.1.3 The role of Platelets

Platelets circulate in the blood as resting discoid shapes as their primary role is in heamostasis and the formation of a pseudoendothelium at a wound site. Platelets trawl along the vascular endothelium inactive until they encounter exposed collagen or fibrinogen and specific integrins at the injured vascular endothelium site, which initiates activation of the platelet (Adam et al 2003). After injury the vascular vessel constricts preventing further blood loss, the coagulation of blood furthers aids the formation of a clot by adding fibrin to the platelet aggregate and helps to activate more platelets (Mangiacapra and Barbato 2013). One theory speculates that the activation state and the expression of key receptors on platelets differ depending upon the proximity to an

atherosclerotic plaque. For example the closer to the vascular plaque there is more activation and aggregation of platelets, with the activation of platelets adding to the inflammatory response and recruitment of other cells (Badimon *et al* 2011).

A resting platelet can be split into 3 zones, the peripheral, the sol-gel and the organelle zone. The peripheral zone is responsible for adhesion and aggregation, whereas the organelle zone contains the dense body of the platelet including platelet granules (Rendu and Brohard-Bohn 2001). The sol-gel zone of the platelet is responsible for the contraction of the platelet and contains the open canalicular system as well as the dense tubule system (Cimmino and Golino 2013). Platelets have a large surface area due to canalicular systems within the platelet. Channels within the open canalicular system serve as transport for substances in and out of the platelet as well as passages for granule release upon platelet activation (Escolar and White 1991).

Platelet activation involves multiple complex signalling cascades initiating shape change from discoid to stellate to amorphously shaped platelets (Davi and Patrono 2008). Upon activation, the formation of platelet filopodia increases surface area and mediates cell-to-cell adhesion. The adherence of platelets to other activated platelets is sustained through the binding of matrix proteins that works to form and stablise the platelet aggregate (Stalker *et al* 2012). Platelet to platelet adhesion is mediated through several platelet integrins (Nieswandt *et al* 2009). The binding of such linker molecules such as collagen, von Willebrands factor (vWF) and fibrinogen to their respective integrins GPIb and αIIbβ3 or GPIIb/IIIa help to stablise the platelet clot (Bennett *et* al 2009). The release of autocrine and paracrine factors from the activated platelet such as the synthesis and release of TXA_2 aids to recruit more platelets and reinforces aggregation (Fig. 1.2).

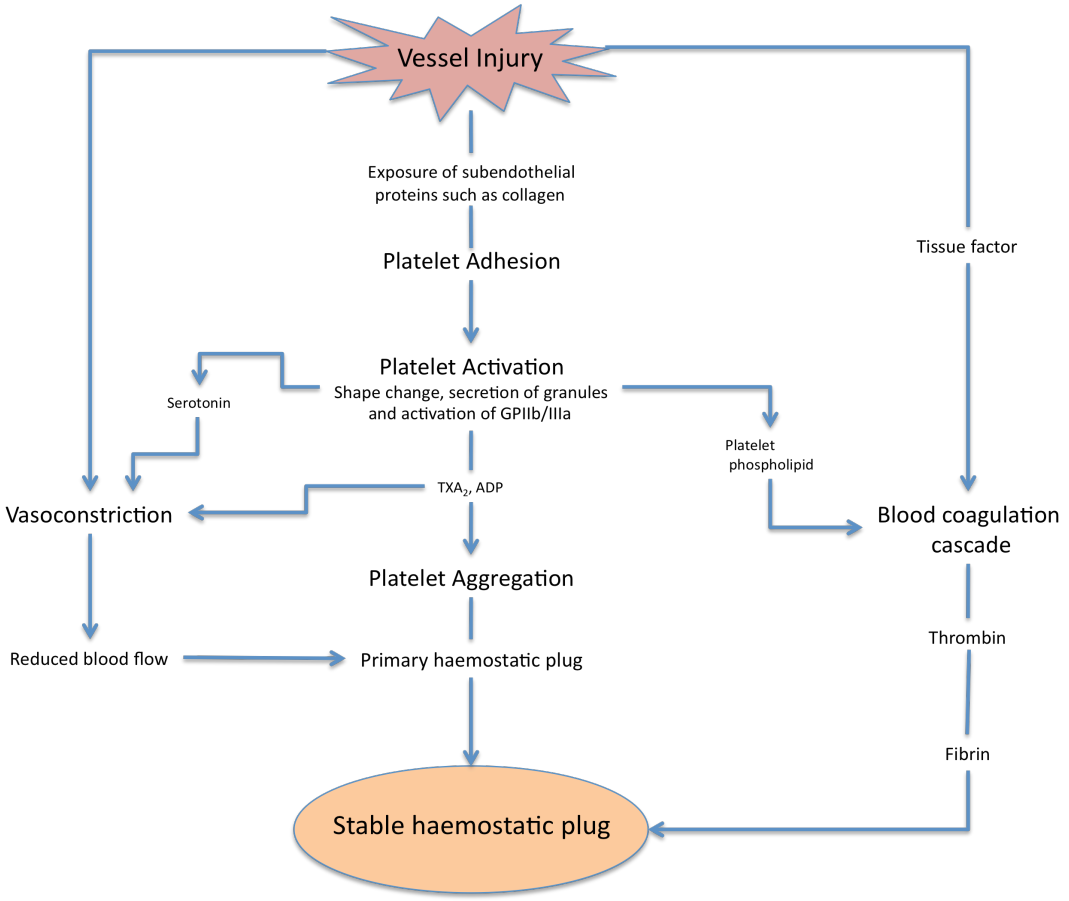


Fig. 1.2:- Overview of the formation of a stable haemostatic plug by platelets. This diagram is adapted from Essential Haemotology 6th Edition, Hoffbrand and Moss.

Upon vascular vessel injury, three processes are initiated, vasconstriction to prevent subsequent blood loss, platelet aggregation and blood coagulation. The damaged vessel exposes subendothelial proteins and platelets trawling the endothelium adhere through specific receptors. Adhesion causes activation of the platelet through intracellular signalling and the release of storage granules further recruiting platelets and adding to the platelet agreggate culminating in a haemostatic plug. Coagulation of the blood adds fibrin and thrombin to further assist platelet activation and to stablise the haemostatic plug.

Platelet activation is one of the many processes that are involved in the cessation of bleeding, termed haemostasis, which there must be a natural balance between procoagulants and anti-coagulant factors. The endothelium has a role in helping to control the necessary balance between all of the cytokines, adhesion factors and the remaining signalling molecules involved throughout haemostasis (Gresele *et al* 2002). Injury or

dysfunction of the vascular endothelium results in exposure of subendothelium matix proteins such as collagen and fibrinogen. Damage to the site also causes a decrease in the localised production of the platelet inhibitors prostacyclin (PGI_2) and Nitric oxide (NO), therefore favouring platelet aggregation. As circulating platelets and the corresponding receptors for fibrinogen and collagen come into contact with their respective ligands, activation is initiated, recruiting more platelets to the site of the injury cultivating in the formation of a clot creating a pseudoendothelium (Fig. 1.3).

There are numerous agonists that can induce platelet activation and aggregation including thrombin, collagen, epinephrine and ADP (Stalker *et al* 2012). The major emphasis in this study is ADP induced platelet activation. Activation of platelets mediated by ADP is through the binding of two surface receptors $P2Y_1$ and $P2Y_{12}$ that consequently initiates complex signalling cascades within the platelet. The release of ADP from platelet granules is essential for the autocrine and paracrine activation of neighbouring platelets, thus providing an important pharmacological target for possible therapeutic intervention.

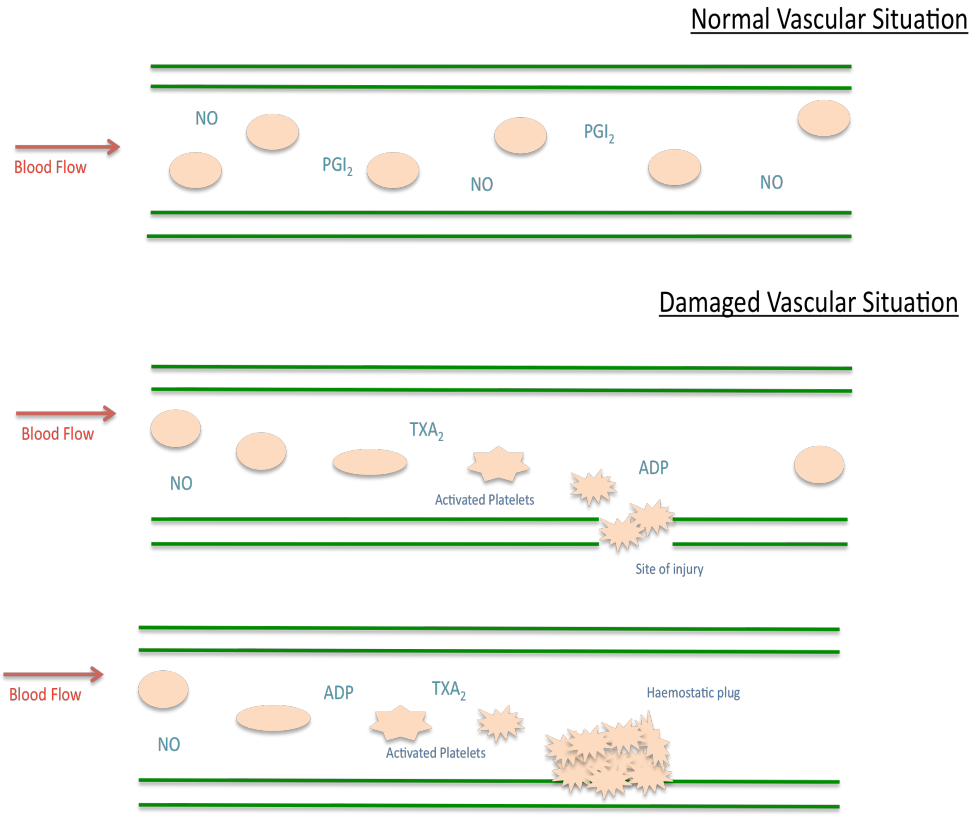


Fig. 1.3:- A simplified schematic highlighting formation of a haemostatic plug due to a vascular injury or the dysfunction of the vascular endothelium. In a normal vascular situation vascular endothelial cells produce nitric oxide (NO) and prostacyclin (PGI_2) inducing intraplatelet cyclic nucleotides inhibiting platelet activation. Upon injury, damaged or dysfunctional endothelium expose subendothelium matrix proteins such as fibrinogen and collagen, activating platelets causing the release of thromboxane A_2 (TXA_2) and adenosine diphosphate (ADP). The release of these chemotatical agents recruits more platelets to the site. Decreased NO and PGI_2, cause a decrease in intraplatelet cAMP and cGMP further assisting the platelet aggregation process to form a haemostatic plug.

1.1.4 Activation of Platelets by Adenosine Diphosphate

The platelet surface contains three receptors for the agonist ADP to induce activation (Jin *et al* 1998). Intracellular signalling induced within the platelet is dependent upon the agonist and the potency of the agonist for the receptor. For instance ATP and ADP can both bind to the same purinergic receptors, however ADP is far more potent than

ATP and initiates distinct signalling pathways within the platelet (Burnstock 2007, Jacobson *et al* 2011). Most platelet receptors are linked to G-proteins that mediate cell signalling between the surface and intracellular messengers (Jurk and Kehrel 2005).

Studies have confirmed that there are at least three receptors for ADP, two of which are G- protein linked (Fontana *et al* 2003). The first being the $P2Y_1$ receptor, which initiates the release of intraplatelet Ca^{2+} stores, whereas the second $P2Y_{12}$ receptor is coupled to the inhibition of the enzyme adenylyl cyclase (AC). The inhibition of AC is essential for a full aggregation response to ADP and the stabilisation of the aggregate (Woulfe *et al* 2001). ADP binds to its respective receptors and initiates cell signalling the end points of which are granule release, shape change and initiation of platelet aggregation. It is suggested that the simultaneous binding of both receptors $P2Y_1$ and $P2Y_{12}$ are required for full aggregation and activation (Erlinge and Burnstock 2008). The ADP-receptor interaction triggers a series of events that leads to aggregation, including a decrease in platelet cyclic nucleotides, the influx and mobilisation of Ca^{2+}, as well as the re-organisation of the platelet cytoskeleton (Savage *et al* 2001). The endpoint of aggregation is the expression of integrins such as GPIIb/IIIa. When a platelet is activated, intrinsic glycoproteins including the GPIIb/IIIa complex, become exposed on the plasma membranes, further assisting the aggregation process (Savage *et al* 2001). Upon activation the contents of the granules found within platelets are released, recruiting more platelets to the site. The nucleotides ADP and ATP are both found within the dense granules, whereas the α granules contain such proteins as fibrinogen that activate more platelets and mediate cell-to-cell adhesion helping to stabilise the forming clot (Davi and Patrono 2008).

The $P2Y_1$ receptor stimulates the hydrolysis of PIP_2 (phosphatidyl inositol bisphosphate) into IP_3 (phosphoinositol triphosphate) and DAG (diacylglycerol) via the enzyme PLCβ (phospholipase C beta) (Fig. 1.4). IP_3 binds to its receptor and generates the release of Ca^{2+} into the intracellular environment. Ca^{2+} and DAG act to activate protein kinase C (PKC) to rearrange the cytoskeleton for sub sequential granule release. The release of granules into the extracellular environment allows 'outside in' signalling and acts to cause conformational changes in integrins to allow the binding of fibrinogen to further initiate aggregation. The ADP receptor $P2Y_{12}$ inhibits the actions of adenylate cyclase therefore suppresses the levels of intracellular cyclic Adenosine Monophosphate (cAMP) (Gachet 1997, Erlinge and Burnstock 2008) (Fig. 1.4). The release of granule

contents from platelets has both an autocrine and paracrine signalling effect. As further platelets are recruited to the site of injury, releasing activators such as TXA_2 and ADP, as well as proteins including fibrinogen that help to reinforce the aggregatory response of the platelet (Packham and Rand 2011, Stalker *et al* 2012). Furthermore the activation of the coagulation system generates thrombin, which additionally activates platelets, with the end point of this pathway producing fibrin, which reinforces and stabilises the aggregated platelets at the site of injury (Rivera *et al* 2009, Stalker *et al* 2012).

Activation of platelets by ADP includes a decrease in cAMP, which is mediated by the inhibition of AC upon the binding of ADP, as well as the action of platelet PDE on endogenous concentrations of cAMP. Platelets contain a second cyclic nucleotide cGMP which is induced by the gaseous messenger NO acting upon soluble guanylyl cyclase (sGC). Circulating NO from either local endothelial cells or platelets act to increase cGMP as well as inducing smooth muscle relaxation. The increase in cGMP can partially reverse platelet shape change as well as inhibit the initiation of platelet aggregation. Localised NO from platelets and the vascular endothelium acts to increase intraplatelet cGMP prohibiting the formation of any more aggregates (Gresele *et al* 2002, Xiang *et al* 2008). An increase in either platelet cGMP or cAMP initiates further signalling. The binding of cGMP to protein kinase G (PKG) or cAMP to protein kinase A (PKA), causes phosphorylation of vasodilator stimulated phosphoprotein (VASP). The phosphorylation of VASP has an important role in the reorganisation of the cytoskeleton, as the phosphorylation of VASP at serine-157, serine-239 or threonine-278, is closely associated with platelet inhibition (Massberg *et al* 2004). These intra-platelet signalling cascades are summarised in Fig.1.4.

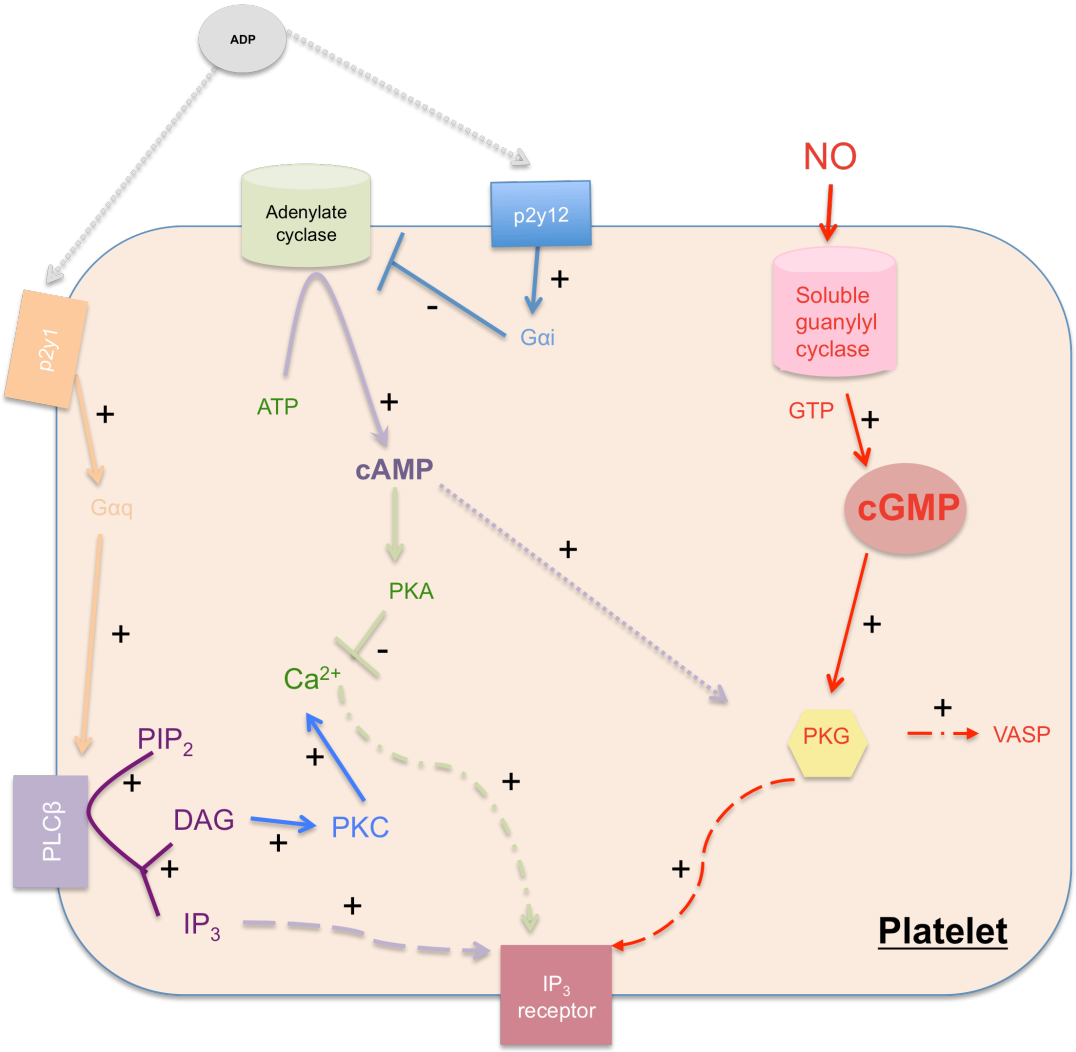


Fig.1.4:- An overview of intracellular platelet signalling upon activation with ADP. ADP binds to its respective receptors $P2Y_1$ and $P2Y_{12}$ on the platelet surface. Simultaneous binding of each receptor is required for aggregation. The ADP-receptor interaction triggers a series of events ultimately leading to aggregation. The $P2Y_1$ receptor stimulates the hydrolysis of PIP_2 (phosphatidyl inositol bisphosphate) into IP_3 (phosphoinositol triphosphate) and DAG (diacylglycerol) via the enzyme PLCβ (phospholipase C beta). IP_3 binds to its receptor and generates the release of calcium ions into the intracellular environment. Ca^{2+} and DAG then act to activate PKC (protein kinase C) to rearrange the cytoskeleton for granule release. The ADP receptor $P2Y_{12}$ inhibits the actions of adenylate cyclase therefore suppresses the synthesis of intracellular cAMP. Nitric oxide acts to increase the amount of cGMP. The increase in cGMP acts to change the platelet shape back to normal whilst also going on to activate further signalling substrates such as protein kinase G (PKG) and vasodilator-stimulated phosphoprotein (VASP).

1.1.5 Inhibitory mechanisms of platelet activation

Upon platelet activation complex cell-signalling cascades are initiated to achieve full shape change and aggregation, (Stalker *et al* 2012). There are several mechanisms that prevent unwanted activation of platelets that induce multiple key molecules that prohibit the aggregation of platelets. These inhibitory molecules include the cyclic nucleotides cGMP and cAMP (Schwarz *et al* 2001). cAMP and cGMP inhibit platelet function through the phosphorylation of key proteins throughout the Ca^{2+} signalling pathway, such as the TXA_2 receptors, and the IP_3 receptor (Cavallini *et al* 1996, Feijge *et al* 2004, Crane *et al* 2005). In conjunction there are numerous endogenous platelet inhibitors, which serve to prevent unwanted activation and aggregation. These include prostglandin E_1 (PGE_1) and prostacyclin I_2 (PCI_2) that each activate AC via the α subunit of the stimulatory G- protein ($G\alpha_s$). AC is potently inhibited by the α subunit of the pertussis toxin-sensitive inhibitory G -protein ($G\alpha_i$). AC catalyses the conversion of ATP into cAMP, which activates PKA and further stimulates downstream signalling, also causing an increase in phophodiesterase 3 (PDE3). An increase in platelet cAMP acts to inhibit platelet activation via the inhibition of Ca^{2+} mobilisation from platelet stores (Feijge *et al* 2004).

Another endogenous inhibitory molecule is nitric oxide (NO), the gaseous messenger potently inhibits platelet aggregation through the direct stimulation of guanylyl cyclase (GC) (Schwarz *et al* 2001, Garthwaite 2010, Naseem and Roberts 2011). There are two forms of guanylyl cyclase, the soluble form, which is activated by NO and a membrane bound particulate form, which is activated by atrial natriuretic factor (ANF) and several different peptides (Francis *et al* 2010, Crane *et al* 2005). There is no evidence to suggest that membrane bound particulate form of the enzyme exists in human platelets (Walter and Gambaryan 2009). Within platelets, soluble guanylyl cyclase, which exists as a heterodimer, needs both subunits for activity and contains a prosthetic haem, which acts as the site for NO interactions (Underbakke *et al* 2013).

NO significantly inhibits platelet aggregation by increasing cGMP, through the direct stimulation of the enzyme soluble guanylyl cyclase (sGC). Soluble guanylyl cyclase is chemically modified upon the binding of NO, with the resulting enzyme catalysing the conversion of guanosine triphosphate (GTP) to cGMP and pyrophosphate enhancing the activation status of sGC (Poulos 2006). cGMP acts as secondary messenger much like

cAMP inducing further signalling within the platelet (Schwarz *et al* 2001). PKG is activated by cGMP, and has similar substrates to PKA such as the phosphorylation of VASP inhibiting platelet aggregation (Massberg *et al* 2004).

Platelets, like other vascular cells can produce reactive nitrogen species (RNS), including peroxynitrite and NO (Valko *et al* 2007). In platelets, RNS can induce a change in intraplatelet Ca^{2+} and can act as secondary signalling messengers in collagen or thrombin induced platelet activation (Olas *et al* 2004). As previously mentioned NO induces an increase in cGMP, predominantly causing the inhibition of intracellular Ca^{2+} release and influx.

NO derived from the vascular endothelium and platelets has several important roles within the vascular system, acting as both an antiplatelet agent and can be pro-aggregatory (Thomas *et al* 2008). Some important roles of NO include inhibiting platelet aggregation, inhibiting platelet adhesion to vascular endothelium as well as further recruitment to formed thrombi, and the development of leukocyte-platelet aggregates (Cooke and Dzau 1997). Although it is well characterized that NO stimulates guanylyl cyclase (Naseem and Roberts 2011), the mechanism in which NO initiates other signalling cascades within platelets is not fully understood. One experimental approach would be to isolate human platelets and expose them to nitric oxide. There are several methods for the chemical synthesis of NO, one common protocol is to use agents that deteriorate at 37°C to produce nitrogenous species. These chemical mimetics induce the NO donors 3-morpholino-sydnonimine (Sin-1) and sodium nitroprusside (SNP), which stimulate the production of cGMP via soluble guanylate cyclase as well as associated signalling.

Cyclic nucleotides are degraded by phosphodisesterases (PDE) converting them into inactive 5'-nucleotide metabolites in a negative feedback loop (Schwarz *et al* 2001). It is difficult to solely obtain the effects of either cyclic nucleotide on platelet aggregation alone, as an increase in one is postulated to influence the concentrations of the other (Siso-Nadal *et al* 2009). For example a rise in cGMP leads to an increase in cAMP through the inhibition of PDE 3 (Fisch *et al* 1995). cGMP and cAMP potently inhibit platelet activation and are one of numerous endogenous molecules that can prevent platelet activation and aggregation. Both cGMP and cAMP pathways utilize similar PDE enzymes for the degradation of the product, therefore theoretically inhibiting any PDE in

experiments both pathways must be examined. This will have to be taken into account in experiments (Fig. 1.5).

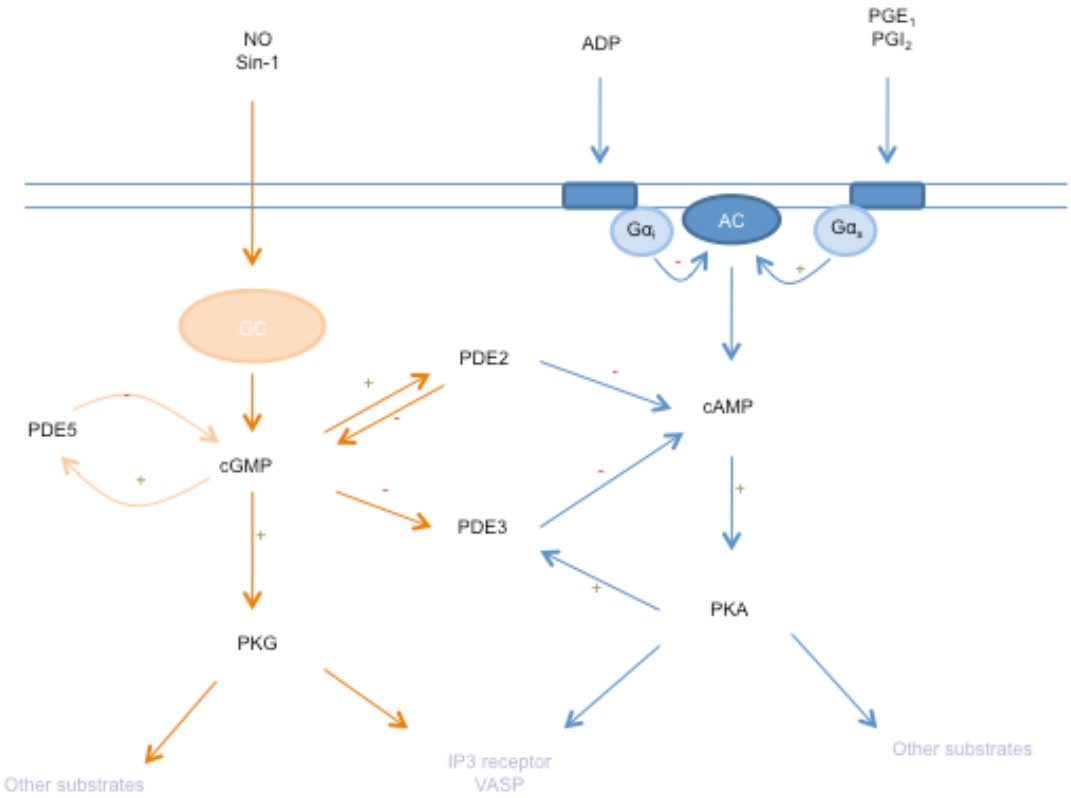


Fig 1.5:- A simplified overview of crosstalk between cAMP, cGMP and respective phosphodiesterases (PDE).

NO binds to soluble guanylyl cyclase (sGC) causing the conversion of GTP to cGMP. cGMP acts as a secondary messenger producing further downstream signalling events, conversing on cGMP protein kinase (PKG) before acting on other substrates. These substrates include the inositol 1,4,5,-triphosphate receptor (IP3 receptor) and vasodilator-stimulated phosphoprotein (VASP). Sin-1 produces NO therefore activates the same signalling cascades.

Prostaglandin E_1 (PGE_1) and prostacyclin (PGI_2) each activate adenylyl cyclase (AC) via the α subunit of the stimulatory G protein ($G\alpha_s$). AC is strongly inhibited by the α subunit of the pertussis toxin-sensitive inhibitory G protein ($G\alpha_i$). AC catalyses the conversion of ATP into cAMP, which activates cAMP protein kinsase (PKA). This in turn stimulates further signalling molecules. PKA causes an increase in PDE3. PDE3 like the other PDE acts in a negative feedback loop to rapidly degrade the cyclic nucleotides converting them into inactive 5'-nucleotide metabolites.

1.1.6 The inhibition of platelets

As platelets are implicated in numerous diseases, including CVD, they serve as a popular pharmacological target and due to the complexity of platelet physiology, provide several potential areas for drug intervention (Cattaneo 2003).

Despite recent advances in anti-platelet medication, aspirin remains the most prescribed in the prevention of recurrent ischemic events (Lordkipanidze 2012). Aspirin inhibits platelet aggregation through the inhibition of COX1, a key enzyme in the synthesis of TXA2 (Kovacs *et al* 2013). Aspirin irreversibly modifies COX, rendering the enzyme inactive for the lifespan of the individual platelet. Due to this inactivation, the arachidonate metabolism and synthesis of TXA_2 is halted and platelet aggregation cannot occur (Patrono *et al* 2008, Kovacs *et al* 2013).

There are several other antiplatelet medications that target different molecules within the platelet, two of which are abciximab and tirofiban. These inhibit platelet aggregation by targeting the binding of fibrinogen, which is the last step in the aggregation process irrelevant of the initial agonist. These drugs target the GPIIb/IIIa complex and are not reliant upon a single pathway within intraplatelet signalling (Patrono *et al* 2008).

The ADP receptors $P2Y_1$ and $P2Y_{12}$ are also targeted medically by the drug clopidogrel, as ADP is an important agonist, due to the nucleotide being released from the dense granules reinforcing aggregation. The binding of both receptors is required for full aggregation to occur, so inhibition of one will prevent aggregation from initiating (Floyd *et al* 2012). To provide the most effective intervention, anti-platelet therapy often uses two of the above anti-platelet drugs, most commonly a combination of aspirin and clopidogrel (Lordkipanidze 2012).

There has been a significant interest into the effects of natural products inhibiting platelet function with evidence for new natural products continuing to emerge. Some products such as alfalfa or nettles inhibit aggregation by eliciting an increase in cGMP (Pierre *et al* 2005), whereas kiwi fruits and tomatoes inhibit aggregation through inhibiting the expression of the integrin GPIIb/IIIa (Duttaroy and Jorgenson 2004, O'Kennedy *et al* 2006). These natural remedies have been shown to target key signalling molecules that are essential to allow platelet aggregation. The majority of studies have been tested with participants taking the natural products as additional supplements. Whether the same

effects are observed from diet alone is therefore questionable (Ostertag *et al* 2010). Evidence nevertheless is continually being produced showing that polyphenol compounds definitively have inhibitory effects upon platelet activation (Michalska *et al* 2010).

The cardioprotective effects of garlic have been widely investigated (Rahman 2007), including the ability of garlic to inhibit platelet aggregation (Rahman and Billington 2000, Rahman and Lowe 2006). There are many different preparations of garlic with most displaying inhibition of platelet aggregation during *in vitro* studies however with varying success *in vivo*. For example an oil extract of garlic inhibited platelet aggregation *in vitro*, but did not inhibit aggregation *in vivo* (Morris *et al* 1995). Fresh garlic preparations displayed an ability to inhibit platelet aggregation both *in vitro* and *in vivo* (Ali and Thompson 1995). Similarly an aged garlic extract (AGE) also displayed inhibitory effects upon platelet activation, despite the fact that fresh garlic and AGE possess different components and properties (Steiner and Lin 1998, Rahman and Billington 2000, Allison *et al* 2006a).

1.2 Aged Garlic Extract

There are many different commercially available preparations of garlic, from its raw form, to garlic capsules. Raw garlic is most commonly known and is used as a flavouring agent, however there are other commercially available preparations marketed for different purposes. For example odourless garlic capsules can be found in most health food stores as a dietary supplement.

The process for each garlic preparation is distinct and unique to the product therefore the components can vary between preparations. One such preparation is Aged Garlic Extract (AGE) and can be found across the globe in health food stores under the commercial name Kyolic (www.kyolic.com). Kyolic is produced by Wakunaga of America Co., Ltd, Mission Viejo, USA, with the garlic extract cultivated from organically grown garlic, it is then subjected to an aging process of up to 20 months. AGE is standardized against one important component, S-allyl-$_{L}$-cysteine (SAC), an organosulphur compound derived from raw garlic, thus providing a way of managing quality control across batches of the garlic extract. The components of AGE differ substantially to that of raw garlic as

they have a higher quantity of organosulphur compounds, and have shown to have no adverse effects on human consumption (Lawson and Gardner 2005).

There has been extensive research into the therapeutic benefits of AGE, the outcomes of these preliminary studies indicate potential health benefits in the areas of cardioprotective properties, enhancement of the immune system, anti-tumourgenic, anti-microbial as well as anti-depressive (Fleischauer *et al* 2000, Kyo *et al* 2001, Steiner and Li 2001, Rahman 2007, Butt *et al* 2009).

Garlic has been identified as a strong antioxidant (Dillon *et al* 2003), as AGE is a preparation of garlic it too has these same benefits (Butt *et al* 2009). There are numerous studies to indicate that AGE can have positive benefits upon health. These include several studies documenting the antioxidant effects of AGE.

An antioxidant can be defined in two ways: In chemistry an antioxidant is a substance, which inhibits oxidation, the second is in biochemistry and relates to an enzyme or substance that is capable of counteracting the damaging effects of oxidation. These definitions are now limited and needs to be challenged, as new studies that refer to an antioxidant often infer different and extended roles. An antioxidant may refer to a chemical reaction where the substance can directly interact with the free radical as previously defined. However, It may now also refer to an effect upon an enzyme, which decreases the presence of a free radical causing an indirect physiological effect. For example a green tea extract has added evidence to this extended role of an antioxidant, as green tea has been shown to directly influence several enzymes causing a decrease in oxidative mediated damage (Frei and Higdon 2003, Basu *et al* 2013, Misaka *et al* 2013).

There are studies that suggest AGE may have both a chemical and physiological effect as an antioxidant. For example AGE has been shown to be effective at both scavenging free radicals as well as altering the production of oxides by influencing intracellular enzymes (Torok *et al* 1994, Ide and lau 1999, Morihara *et al* 2011). Studies appertaining to the effects of AGE upon cellular activity include both *in vivo* and *ex vivo* investigations. For example, *ex vivo* studies have indicated that AGE can affect numerous cell types as the extract may scavenge free radicals and interfere with cell signalling (Dillon *et al* 2003, Allison *et al* 2006a, Morihara *et al* 2011). The positive outcomes of these preliminary *in vivo* and *ex vivo* studies indicate that there is potential for the garlic extract to be beneficial to immunological reactions and atherosclerosis (Table 1.1).

The increased glycation of proteins due to hyperglycaemia, is a major problem in those with diabetes. *In vitro* studies have indicated that AGE inhibited the formation of advanced glycation end products (AGEPs), which are associated with the pathogenesis of diabetes. Such results indicate that AGE may help to prevent the formation of AGEPs and associated complications in diabetes (Ahmad and Ahmed 2006, Ahmad *et al* 2007) (Table 1.1).

Study	Outcome of study	Study Designs
Steiner and Lin 1998	Reduced blood pressure	*In vivo*, 10 intervention month trial in male subjects
Ide and Lau 1999	AGE scavenged superoxides and NO as well as protected endothelial cells from LDL induced injury	*In vitro* study using bovine pulmonary artery endothelial cells
Rahman and Billington 2000	AGE inhibits ADP induced platelet aggregation *in vivo*	*In vivo* trial, 13 week with n=23 (male and female)
Campbell *et al* 2001	Antiatherogenic effects by inhibiting smooth muscle proliferation as well as the accumulation of lipids on arterial walls	*In vivo* rabbit model study, 4 groups with n=6 per group
Kyo *et al* 2001	AGE displayed immunodulatory effects mice across several immune cells	*In vivo* mouse models, with average results representing n=10
Steiner and Li 2001	Inhibition of platelet aggregation	Randomised double blind *in vivo* 6 week trial, n=34 (male and female)
Dillon *et al* 2003	Inhibited the oxidation of LDL *in vitro*	*In vitro* study using isolated human LDL
Ahmad and Ahmed 2006, Ahmad *et al* 2007	Inhibited the formation of Advanced glycation end products, an end product associated with diabetes	*In vitro* studies using glycated bovine serum albumin
Allison *et al* 2006a	AGE decreases platelet Ca^{2+}	*In vitro* study using isolated human platelets
Allison *et al* 2006b	Preliminary results suggest that AGE increases cAMP	*In vitro* study using isolated human platelets
Morihara *et al* 2011	Inhibited the production of superoxides in human neutrophils	*In vitro* study using isolated human neutrophils
Allison *et al* 2012	A decreased expression of the GPIIb/IIIa receptor was observed in platelets treated with AGE	*In vitro* study using isolated human platelets

Table 1.1:- Summary of a number of studies and their outcomes investigating the effect of AGE in different cell types . Sample size is provided where available.

AGE has also displayed anti-atherogenic effects inhibiting smooth muscle proliferation as well as inhibiting the accumulation of lipids on the arterial cell wall (Campbell *et al* 2001). Across numerous *in vivo* and *in vitro* studies AGE has been shown to be efficient at reducing some of the parameters that are associated with CVD including lowering blood pressure *in vivo* (Steiner and Lin 1996) and the inhibition of agonist induced platelet aggregation *in vitro* (Rahman and Billington 2000, Steiner and Li 2001).

These early observations warrant further investigation to determine the mechanisms of inhibition of platelet aggregation by AGE.

1.2.1 The effects of AGE on Platelet Signalling

Previous investigations have provided substantial evidence to indicate that AGE inhibits ADP induced platelet aggregation, through influencing several key signalling molecules (Rahman and Billington 2000, Allison *et al* 2006a). Platelet activation is a very complex process, and offers multiple targets by which AGE may act to inhibit aggregation.

One of the key pathways in platelets is the production of TXA_2, which is dependent upon AA and COX activity (Stalker *et al* 2012). When released from the platelet TXA2 has autocrine and paracrine function and is metabolized to TXB_2, whereas $PCI_2$is metabolized to 6-keto $PGF_{1\alpha}$ and TXB_2. Work by Rahman and Billington suggests that these eicosanoids PCI_2 and TXA_2 are not key targets of AGE and are not the cause of inhibition of platelet aggregation by AGE. Further research analysing other biochemical components within the ADP signalling pathway was investigated, with one such target being the influx of Ca^{2+} into the platelet.

The mobilisation and influx of Ca^{2+} is an important process in ADP induced platelet activation; decreases in platelet Ca^{2+} will result in the inhibition of aggregation. AGE was shown to decrease platelet Ca^{2+} by either suppressing the mobilisation of Ca^{2+} or by chelating the ion (Allison *et al* 2006b).

Platelet Ca^{2+} is a key molecule that is influenced by a number of other intraplatelet signalling cascades including the secondary messenger cAMP. Previous studies (Allison *et al* 2006a, Allison *et al* 2012) indicated that AGE also significantly increased platelet cAMP, an endogenous inhibitory molecule that is normally degraded upon platelet activation with ADP. As AGE can influence both cAMP and Ca^{2+} it is possible that the mechanism of inhibition is multi mechanistic with AGE targeting several platelet molecules. This is initial evidence that AGE may be multimechanistic as AGE targets both Ca^{2+} and cAMP. Previous investigations indicate that a further target of AGE is the binding of platelets to fibrinogen.

The end point of platelet activation is the expression of the important integrin GPIIb/IIIa. The expression of this integrin is important for its interaction with fibrinogen

and thus can provide an important marker for platelet activation, as no expression of the glycoprotein indicates there is no activation of platelets. The binding of fibrinogen is dependent upon the expression of GPIIb/IIIa on the platelet surface and this is an endpoint of platelet activation. Platelets treated with AGE displayed a diminished expression of the receptor despite activation by ADP (Allison *et al* 2012). Initial studies indicate that AGE has a role in inhibiting the expression of GPIIb/IIIa following ADP activation of isolated human platelets, this decrease in the fibrinogen receptor indicates another possible mechanism of inhibition of platelet aggregation induced by AGE (Allison *et al* 2012).

There is evidence to support the commonly held belief that garlic can positively influence some of the risk factors associated with CVD (Rahman and Lowe 2006, Butt *et al* 2009), one of which is the hyper activation of platelets (Projahn and Koenen 2012, Shulz and Massberg 2012). Understanding how AGE or its components effect platelet activation may provide potential novel pharmodynamic targets for therapeutic intervention. This study aims to elaborate on previous findings to investigate the effect of AGE upon intraplatelet signalling. Identifying potential targets for AGE within platelets will add to established scientific evidence that AGE can inhibit platelet activation, and may provide an alternative therapy for those with disorders that include the hyper activation of platelets. This study will largely focus on the effects of whole AGE on NO and sGC and the interdependence of cAMP and cGMP.

1.3 Justification of study

Previous studies have investigated the effect of AGE upon cAMP, however the effect of AGE upon the second cyclic nucleotide cGMP is unknown. There are no studies that have looked at the effect of AGE upon cGMP, however previous investigations using other phytochemicals suggest that cGMP may be a target for inhibiting platelet function (Pierre *et al* 2005, Allison *et al* 2006a).

The interdependence of cyclic nucleotides on each other is widely speculated. As AGE can influence cAMP, the effect of cGMP and cAMP on the opposite nucleotide and whether this is a major or minor effect will also be investigated. Identifying the effect of

AGE upon important signalling molecules and the mechanism by which AGE inhibits platelet aggregation has the potential to provide a new pharmaceutical target for therapeutic intervention. This provides a strong justification to investigate the effects of AGE upon intraplatelet signalling.

Other phytochemicals have been shown to target cGMP, as there is evidence that AGE already targets cAMP it is of high interest to investigate the effect of AGE upon cGMP. Establishing the effect of AGE upon both cAMP and cGMP will add to the mechanisms of inhibition of platelet aggregation by AGE, and will provide more evidence that AGE should be taken as supplement to help prevent the occurrence of CVD.

1.4 Approach to Study

This study will investigate the potential of AGE to modulate platelet functions with specific reference to NO and cyclic nucleotide signalling. Previous work (Allison 2007) has identified that AGE can influence intraplatelet signalling molecules such as Ca^{2+} as well as decreasing the expression of the fibrinogen receptor GPIIb/IIIa (Allison *et al* 2006b, Allison *et al* 2012). Previous studies indicated that AGE could increase intraplatelet cAMP, however the effect of AGE upon cGMP is largely unknown and warrants further investigation (Allison *et al* 2006a, Allison *et al* 2012). This study aims to identify the full inhibitory effects of AGE upon platelets and corresponding functions, by investigating whether AGE can modify intraplatelet cyclic nucleotide signalling.

The effect of AGE upon platelets will be investigated first to confirm other studies that indicated that AGE could inhibit ADP induced platelet aggregation. These initial studies will provide proof of principle and corroborate previous findings that AGE can inhibit platelet aggregation as well as substantiate data that AGE increases intraplatelet cAMP.

The effect of AGE upon intraplatelet cGMP will be established and directly quantified using aggregation and specific inhibitors to probe sGC and associated PDE. The nucleotide was directly quantified using Sin-1 as a stimulant. Different concentrations of AGE will be investigated in aggregation studies and when quantifying cGMP to establish the minimum dosage to provoke inhibition of platelet activation. This is a unique investigation, as previous studies have not used such low concentrations of AGE. Platelets

will also be imaged using scanning electron microscopy to study the effects on the overall platelet in response to specific stimuli.

To establish the effects of AGE on cGMP and assocociated signalling, human platelets will be isolated and the effect of AGE and multiple inhibitors will be investigated using aggregometry, a light transmission method. In parallel platelets will be extracted and biomarkers investigated including intraplatelet cAMP and cGMP. To identify the role of specific signalling molecules within the platelet and their response to treatment with AGE, the following specific inhibitors will be used with their point of action highlighted in Fig. 1.6:

- ODQ:- The inhibitor ODQ inhibits the enzyme sGC, preventing synthesis of cGMP induced by NO.
- SQ 22536:- SQ 22536 inhibits adenylate cyclase, preventing the induction of cAMP and thus preventing further signalling induced by the nucleotide.
- Zaprinast:- The inhibitor Zaprinast inhibits cGMP specific PDE5 thus allowing cGMP to accumulate within the platelet.
- IBMX:- Platelet PDE will be inhibited using IBMX, a non-specific inhibitor of PDE, allowing both cyclic nucleotides to accumulate and the effect upon either to be quantified without degradation.

To identify the effects of NO on platelet function and whether AGE can modify NO signalling and respective NO metabolites such as nitrite and nitrotyrosine, NO donors will be utilized. There are many nitric oxide donors currently on the market, with each having a different mechanism of producing NO with the optimum dosage often dependent upon the cell line (Kevil *et al* 2011). The chemical mimetics 3-morpholino-sydnonimine (Sin-1) and sodium nitroprusside (SNP) generate NO causing stimulation of ssGC to induce synthesis of the secondary signalling molecule cGMP (Gerzer *et al* 1988, Fisch *et al* 1995, Garthwaite 2010). Sin-1 is a non enzymatic NO donor and can spontaneously release NO in solution as well generating the superoxide peroxynitrite ($ONOO^-$). To establish whether the inhibitors are functioning correctly within the signalling system, each will be tested with a specific stimulus. For example, if ODQ has bound to sGC, the increasing effects of Sin-1 on cGMP should be reversed (Fig. 1.6).

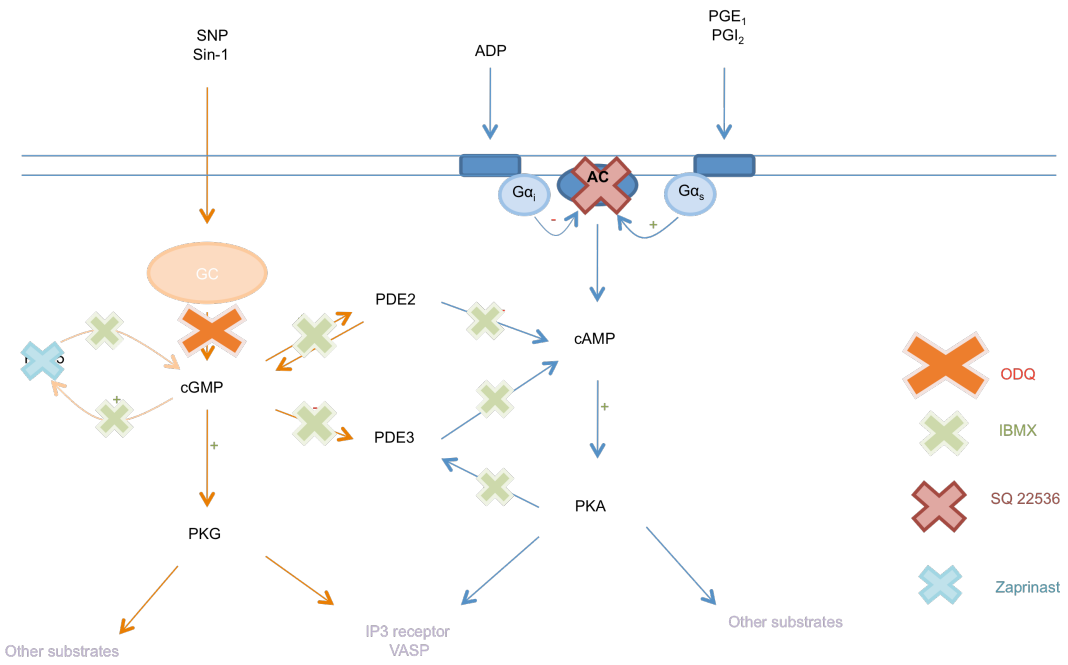


Fig 1.6. :- Overview of cyclic nucleotide signalling in platelets with inhibitors. X indicates where each respective inhibitor is acting. NO binds to soluble guanylyl cyclase (sGC) causing the conversion of GTP to cGMP. cGMP acts as a secondary messenger producing further downstream signalling events, conversing on cGMP protein kinase (PKG) before acting on other substrates. These substrates include the inositol 1,4,5,-triphosphate receptor (IP3 receptor) and vasodilator-stimulated phosphoprotein (VASP). Sin-1 and SNP produce NO therefore activates the same signaling cascades. ODQ inhibits sGC, preventing activation of cGMP by NO. AC catalyses the conversion of ATP into cAMP, which further activates cAMP protein kinase (PKA). This in turn stimulates other downstream signalling molecules. SQ 22536 inhibits adenylate cyclase, preventing further induction of cAMP and thus preventing signalling. PKA causes an increase in PDE3, which like the other PDE acts in a negative feedback loop to rapidly degrade the cyclic nucleotides converting them into inactive 5'-nucleotide metabolites. IBMX is a non-specific inhibitor of PDE, allowing the cyclic nucleotides to accumulate and the effect upon either to be visualised without degradation. Zaprinast inhibits cGMP specific PDE5 thus allowing cGMP to accumulate.

An overview of the approach taken to investigate the effect of AGE upon platelet NO and cyclic nucleotide signalling is presented in Fig. 1.7.

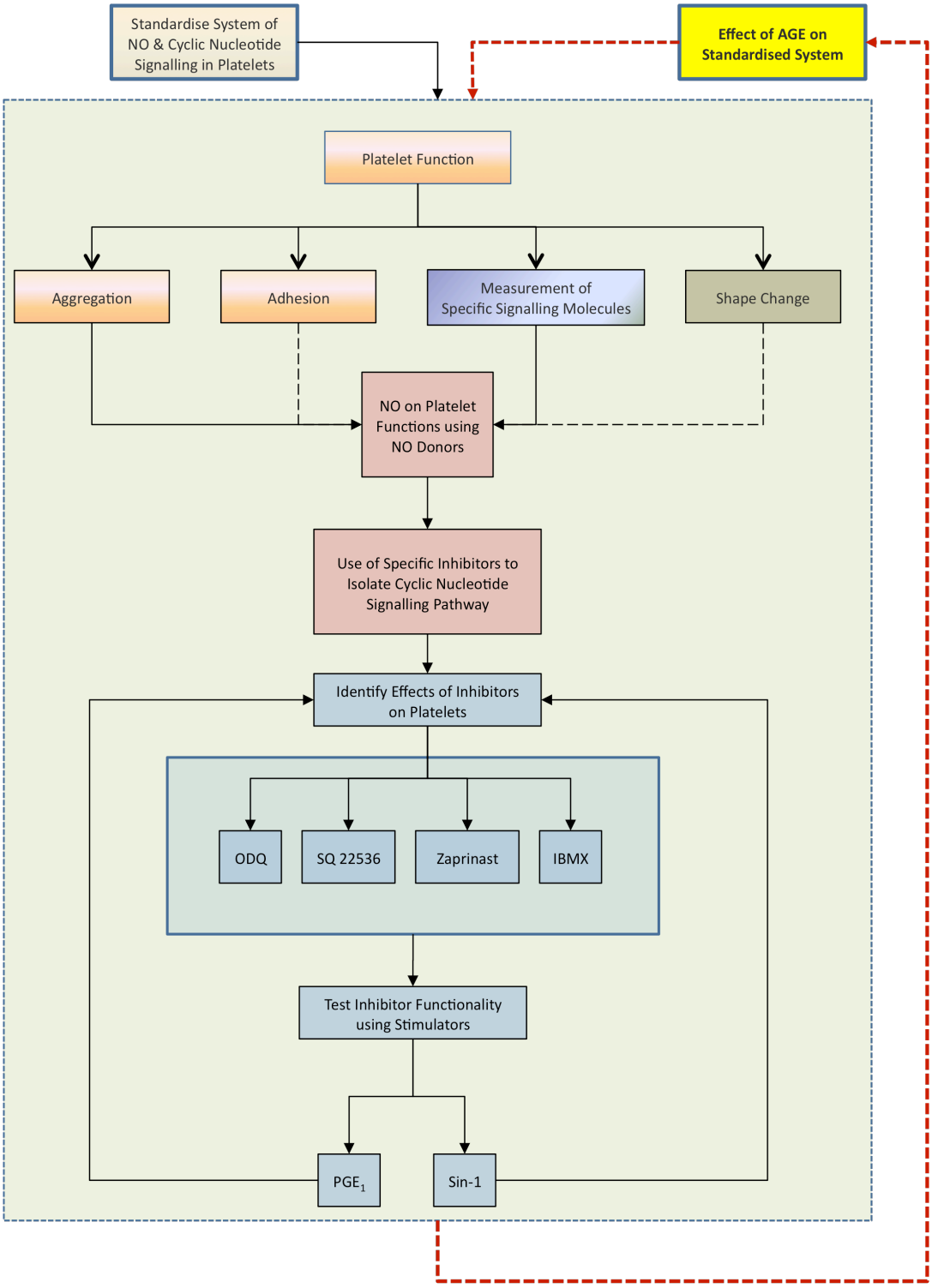


Fig 1.7: Overview of the approach of this study. The platelet NO and cyclic nucleotide signalling system will first be established with the role of specific components identified using several inhibitors and investigating different platelet functions. Once the system and inhibitors have been validated to be working efficiently, the same intraplatelet signalling components will be investigated in response to treatment with AGE.

1.5 Aims and Objectives

As two important signalling molecules within the platelet system, the effect of AGE upon NO and intraplatelet cGMP were investigated.

The aim of this study was to establish the effect of AGE upon intraplatelet cGMP and associated signalling.

This aim will be achieved through two main objectives:

- Identifying the role of NO, cAMP and cGMP within platelets using specific inhibitors (Fig. 1.6) and their effects upon platelet function, including ADP induced platelet shape change. This is to establish the functionality of the signalling system.

- Having established the functionality of the inhibitors, the effect of AGE upon the same platelet functions and the signalling molecules NO, cAMP and cGMP will be investigated.

1.5.1 Hypothesis

AGE will modify NO signalling and intraplatelet cyclic nucleotides

2 Materials and Methods

2.1 Materials

2.1.1 Materials list

BDH Laboratory Supplies UK: Acetic Acid; Diethyl Ether; Methanol

Bioline: HYPERPAGE prestained protein marker

Cell Signalling Technologies: Goat anti-rabbit HRP IgG antibody; β-actin monoclonal rabbit antibody; GAPDH monoclonal rabbit antibody

GE Healthcare: cAMP EIA kit; cGMP EIA kit; ECL film

Invitrogen, UK: EGTA; Goat anti-mouse IgG Alexa Flour 488 antibody; NOVEX prestained marker; PBS tablets; Prolong Gold anti-fade reagent

Merck Biosciences: Fibrinogen; IBMX; Nitric Oxide assay kit fluorometric kit; PGE_1; PGI_2; Thrombin

Santa Cruz: Anti-mouse IgG secondary antibody HRP conjugated; Luminol enzyme substrate developing solutions; Monoclonal mouse Nitrotyrosine antibody

Sigma: ADP; ATP; HEPES; Kodak Fixer / Replenisher; Kodak Developer; Monoclonal anti-actin mouse FITC conjugated antibody; SOD

Tocris Biosciences: GR 144053; SQ 22536; ODQ; Zaprinast

2.1.2 Major equipment used

Biodata Corporation PAP-4D Platelet Aggregometer Profiler

Varian Cary Eclipse fluorescent spectrophotometer

Zeiss, Laser Scanning Confocal microscope model 510 META

Olympus BX51 fluorescent microscope

FEI Inspect S low vacuum Scanning Electron Microscope

Emitech k550x gold sputter coater

BioRad Gel Doc XR

Biorad Chemi Doc XRS

Spectra Max 190 Plate Reader

2.2 Ethical approval and volunteer selection criteria

Ethical approval was granted for this study by both the Research Degree and the University Ethics Committee at Liverpool John Moores University (Reference; 09/PBS/005, July 2009). Informed written consent was obtained from all volunteers prior to venepuncture by the signing of a consent form (Appendix I). Both verbal and a written explanation of the study, was provided. This included a brief explanation of the use of platelets in the study as well as an assurance that no other biological material would be stored or used in any other investigations.

Blood samples (10 - 20 ml) were collected from healthy volunteers, with an age range between 19-65 years, who were known not to be on anticoagulant therapy or any other medication that may interfere with the platelet count or activity. Exclusion criteria for volunteers included those who had taken medication such as aspirin or paracetemol within 24 hrs prior to the blood sample being taken. Those with medical problems that could affect platelet activity were also excluded; this included diabetics as well as those with abnormal haemostasis. After platelets were isolated from the blood sample, the remaining blood cells were destroyed by either chemical disinfection or autoclaving before being discarded.

2.3 Aged garlic extract

The same batch of AGE (Batch Number:- 6E02C, March 2009) was used in all *in vitro* studies and was kindly obtained from Wakunaga of America Ltd, (Mission Viejo, CA). This commercially available garlic extract is prepared in the following manner: Raw garlic is soaked in 15-20% (v/v) aqueous ethanol at room temperature for approximately 20 months to produce Aged Garlic Extract. The extract is then filtered and concentrated under reduced pressure at low temperatures, with the presence of water-soluble compounds found in AGE relatively high when compared to oil-soluble compounds, which is low.

Varying concentrations of AGE typically between 0.1 - 25 % (v/v) were used in experimentation. When necessary the extract was prepared using PBS at room temperature for use in experiments to achieve the desired concentration.

AGE was stored at 4°C in opaque bottles and once opened was discarded after 8 weeks, with another bottle from the same batch then utilised. Per batch of AGE is subject to rigorous quality control inspections to ensure each bottle is standardised to contain the same ratio of ingredients.

2.3.1 Diethyl ether extract of AGE

AGE was found to interfere with a number of assays due its dark colour, especially photometric methods in the visible and UV range between 300 – 700nm. In certain experiments where AGE was known to interfere with assays due to its dark colouring, a diethyl ether extract was required. Although this contained water-soluble components of AGE, it was not as potent as the parent compound.

To prepare this extract, 1 ml of AGE was mixed with 2 ml of diethyl ether, and left to separate in a clean glass universal. The top layer of the separated solution was removed and dried under gaseous oxygen free nitrogen and the dried extract was reconstituted in 1 ml phosphate buffered saline (PBS 10 mM phosphate, 150 mM sodium chloride, pH 7.4) (Dillon *et al* 2003).

2.4 Preparation of platelet suspensions

2.4.1 Preparation of platelet rich plasma

Blood was taken intravenously from the median cubital vein either via a butterfly or 21' gauge needle and placed into anti-coagulant (3.8 % (w/v) trisodium citrate) at a ratio of 9:1 v/v (9 parts blood to 1 part anticoagulant). Platelet rich plasma (PRP) was prepared via centrifugation at 100 x g for 15 min at room temperature (RT). The separated upper plasma layer was removed and placed into plastic container or plastic universal tube. Platelet poor plasma (PPP) was prepared through further centrifugation of the remaining blood sample at 530 x g for 20 min at RT. The separated upper PPP layer was aliquotted into separate plastic containment. PRP was diluted to a 1/100 dilution using PBS (10 mM phosphate, 150 mM sodium chloride, pH 7.4) and platelets were counted using a neubauer haemocytometer. The cell count was adjusted to 2.5 ± 0.5 x 10^5 cells/ml with PPP.

2.4.2 Preparation of washed platelet suspension

PRP was centrifuged for 15 minutes at 1000 x g. and the pelleted platelets were resuspended in HEPES tyrode's buffer (138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 1mg/ml glucose, 20 mM HEPES, pH 7.4) and further centrifuged. The resulting platelet pellet was resuspended in HEPES tyrode's buffer and the count adjusted to approximately 2.5 x 10^5 per ml.

2.5 Platelet aggregation studies

2.5.1 Measurement of ADP induced aggregation

Platelet aggregation was measured using a Platelet aggregation profiler, PAP-4D (Bio/Data Corporation), which utilises a light transmission method to measure platelet aggregation. When platelet aggregation was initiated by the addition of an agonist, the turbidity of the solution decreases allowing more light to reach a photocell detector, with the increase in light detected indicating a higher percentage of platelet aggregation. PRP is continuously stirred throughout testing to prevent platelets settling at the bottom of the glass tubing, which would cause an increase in the optical density of the solution giving a false indication of the percentage of platelet aggregation.

The baseline was calibrated using PPP, aggregation was stimulated in PRP by the addition of 8 µM ADP (final concentration) to make a total volume of 0.2 ml or 0.5 ml, these allowed for the volumes of PRP to be adjusted for low concentrations of either stimulators or inhibitors being investigated. Dilution vehicles including DMSO and ethanol were used as controls to ensure there was no interaction between platelets and the vehicle with concentrations of the vehicle consistently ≤ 0.5 % (v/v). These were prepared through serial dilutions using PBS, ensuring minimal DMSO or ethanol was present.

Each aggregation profile was allowed to run for a total of 4 minutes with several parameters measured including total percentage aggregation and the rates of aggregation. ADP induced aggregation is biphasic, consequently both the primary and secondary rates aggregation were calculated from the traces obtained from aggregation profiles the rates of the primary and secondary phases were determined from the PAP-4D. Control platelets were deemed viable if total percentage aggregation using 8 µM ADP was observed to be ≥ 65%.

2.5.2 The Inhibition of ADP induced aggregation by AGE

The inhibition of ADP induced aggregation by AGE has previously been observed by a number of groups (Rahman and Billington 2000, Steiner and Li 2001, Allison *et al* 2006b). To provide proof of principle and to ensure the same effects were observed with this batch of AGE, platelet aggregation after treatment with AGE was investigated.

Concentrations of AGE from neat liquid form, were prepared from neat AGE liquid with a working range between 0 - 10 % (v/v) respectively. PRP was incubated with each respective AGE concentration at 37 °C for a minimum of 1 minute before aggregation was initiated with 8 µM ADP (final concentration). Each aggregation plot was analysed for total percentage aggregation as well as rates of aggregation for both primary and secondary phases.

2.5.3 The Inhibition of ADP induced platelet aggregation after treatment with chemical inhibitors

In an effort to locate and evaluate specific areas of platelet cell signalling, a variety of chemical agents were employed after dose dependent investigations were undertaken (Table 2.1). Vehicle controls utilised an appropriate diluted amount of the vehicle in which the inhibitor was solubilised. Inhibitors were added to PRP at each respective final concentration and incubated at 37 °C for different time periods, dependent on the inhibitor used (Table 2.1). Aggregation was then initiated via the addition of 8 µM ADP (final concentration) and the reaction was allowed to proceed for 4 mins. Each aggregation plot was analysed for total percentage aggregation as well as rates of aggregation for both primary and secondary phases.

The inhibitors SQ 22536 and ODQ were used to inhibit the enzymes adenylyl cyclase (AC) and soluble guanylyl cyclase (sGC) respectively (Salzman *et al* 1978, Crane *et al* 2005), whereas IBMX and Zaprinast targeted platelet PDE (Haslam *et al* 1999, Hollbrook and Coker 1991). The inhibitor GR 144053 targeted the glycoprotein receptor GPIIb/IIIa (Eldred *et al* 1994, Kirsch and Groot 2002). The chosen inhibitors were used to inhibit important signalling targets with the platelet, using these specific inhibitors the pharmadynamics of inhibition of these signalling mechanisms may help determine the point of action of AGE within the platelet (Table 2.1).

2.5.4 Chemical agents that modify platelet cyclic nucleotides

Aggregation was inhibited using a variety of chemical mimetics that stimulate the production of the intraplatelet cyclic nucleotides. NO donors Sin-1 and SNP acted to increase cGMP, whereas PGE_1 increased intraplatelet cAMP. Concentrations of each stimulator was first determined through dose dependent studies (Table 2.2). To ensure there was no influence from the vehicle in which the stimulator was solubilised (Table 2.2), series dilutions of the vehicles were prepared and investigated as the vehicle control. Stimulators were added to PRP at each respective final concentration and incubated at 37 °C for 1 min. Aggregation was initiated by the addition of 8 µM ADP (final concentration) and the reaction was allowed to proceed for 4 mins. Each aggregation plot was analysed for total percentage aggregation as well as primary and secondary rates of aggregation.

Inhibitor	Concentration (final)	Incubation Time at 37°C	Vehicle	Inhibitor Target, Concentration and Effect of Vehicle
SQ 22536	100 µM	10 min	H_20	Adenylate Cyclase, Concentration recommended by manufacturer (Tocris Bioscience, UK) and Salzman *et al* 1978, No vehicle effect
ODQ	100 µM	10 min	DMSO	Guanylate Cyclase, Concentration taken from Fisch *et al* 1995 and Garthwaite *et al* 1995, DMSO final concentration < 0.5% to ensure no effect
IBMX	100 µM	30 min	DMSO	Non-specific PDE inhibitor, Concentration used from Burrell *et al* 2008, DMSO final concentration < 0.5 % to ensure no effect
Zaprinast	0.76 µM	10 min	DMSO	PDE5 Inhibitor, Concentration as per manufacturers recommendations (Tocris Bioscience, UK), DMSO final concentration < 0.5 % to ensure no effect
SOD	10 Units	5 min	PBS	Removes superoxide anions, Concentration as per manufacturers recommendations (Sigma Aldich, UK), No vehicle effect
GR - 144053	1 µM	5 min	H_20	GPIIb/IIIa receptor inhibitor, Concentration as per manufacturers recommendations (Tocris Biosciences, UK), No vehicle effect

Table 2.1:- Final inhibitor concentrations and their respective solubility and target within platelets. The use of these inhibitors will help determine the mechanisms of action of AGE upon intraplatelet signalling.

Stimulator	Concentration (final)	Incubation Time at 37°C	Vehicle	Inhibitor Target, Concentration and Effect of Vehicle
Sin-1	8 µM	1 min	H_20	NO donor - increases cGMP, also generates peroxynitrite, Concentration determined through dose dependence study, No vehicle effect,
SNP	20 µM	1 min	H_20	NO donor - increases cGMP, Concentration determined through dose dependence study, No vehicle effect
PGE_1	10 nM	1 min	EtOH	Increase cAMP through AC, Concentration determined through some dose dependent studies as well as recommended by Salzman *et al* 1978, Final concentration of EtOH was < 0.5 %

Table 2.2:- Stimulator incubation times, concentrations and solubility along with their respective target within platelets. These agents will help elucidate the effect of AGE upon platelet cyclic nucleotide signalling.

2.6 Determining of intraplatelet cyclic nucleotide concentrations

Intraplatelet cyclic nucleotide concentrations were determined using a readily available competitive enzyme immunosorbent binding assay (EIA) (GE Healthcare) for both cAMP and cGMP.

The EIA uses competition between cyclic nucleotides contained within the sample, against peroxidase conjugated cyclic nucleotides, which are added during the experiment. To achieve this, rabbit anti-cAMP or anti-cGMP antibodies bind to the pre-coated donkey anti-rabbit wells on the 96-well plate. During the assay, cyclic nucleotides within the sample compete against the added peroxidase conjugated cyclic nucleotide for binding to the rabbit anti-cAMP or anti-cGMP antibody. Substrate TMB is added for colour development and read using a plate reader at 450nm with the developed colour inversely associated with the amount of cyclic nucleotide within the sample.

Each cyclic nucleotide was quantified in the presence of the inhibitors ODQ, SQ 22536 and the stimulators Sin-1 and PGE_1. Cyclic nucleotides were also quantified after treatment with 1 % or 5 % (v/v) AGE. Due the relatively low concentrations of cyclic nucleotides within platelets, both cAMP and cGMP were quantified in the presence and absence of the PDE inhibitor IBMX. Experiments also investigated the concentrations of cAMP and cGMP in both an inactive resting state and in platelets activated by ADP.

2.6.1 Determining platelet cGMP / cAMP concentrations in the presence of a PDE inhibitor

Briefly after washing as previously detailed (2.4.2), isolated platelets at approximately 3 x 10^8/ml in HEPES Tyrodes buffer (138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 1mg/ml glucose, 20 mM HEPES, pH 7.4), were incubated with 100 µM IBMX (final concentration) for 30 min. Platelets were further incubated with either 100 µM SQ22536 (final concentration), 100 µM ODQ (final concentration) or PBS for a further 10 mins. This was followed by platelets being treated with either 1 % or 5 % AGE (v/v) (final concentration), 8 µM Sin-1 (final concentration), 10 nM PGE_1 (final

concentration), or a combination of the respective reagents at their final concentrations for 1 minute.

Platelets were either activated with 8 µM ADP (final concentration) for a maximum of 15 seconds before being placed directly onto ice or left in a resting state. Inactivated platelet samples were placed straight onto ice for 5 mins to stop the reaction. Samples were centrifuged at 1000 x g to remove any residual colour and resuspended in lysis buffer as provided with the EIA kit. The manufacturers protocol was followed and a standard curve was constructed (Appendix II, III) with the eventual resulting colour change read at 450nm (spectramax 190 plate reader, softmax pro v5).

2.6.2 Determining platelet cGMP / cAMP concentrations without IBMX

Isolated platelets were washed as previously described (2.4.2) with HEPES Tyrodes buffer (138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 1mg/ml glucose, 20 mM HEPES, pH 7.4), with an approximate count of 3 x 10^8/ml. Platelets were then incubated with either 100 µM SQ22536 (final concentration), 100 µM ODQ (final concentration) or PBS for 10 mins. This was followed by treatment with 1 % or 5 % AGE (v/v) (final concentration), 8 µM Sin-1 (final concentration), 10 nM PGE_1 (final concentration), or a combination of the respective reagents at their final concentrations for 1 minute.

Platelets were either activated with 8 µM ADP (final concentration) for a maximum of 15 seconds before being placed directly onto ice. Inactive platelet samples were placed straight onto ice for 5 mins to stop the reaction. Samples were centrifuged at 1000 x g to remove any residual colour and resuspended in lysis buffer as provided with the EIA kit. The manufacturers protocol was followed and a standard curve was constructed (Appendix II, III) and the eventual resulting colour change was read at 450nm (spectramax 190 plate reader, softmax pro v5).

2.7 Disaggregation studies

2.7.1 The disaggregation of platelets

The effect of the inhibiting reagents Sin-1, PGE_1 and AGE upon pre-aggregated platelets was investigated, to establish whether the disaggregation of platelets could be induced. The NO donor Sin-1, PGE_1 and AGE at various concentrations ranging from 1 – 10 % (v/v), were added to pre-aggregated platelets induced by ADP.

- These experiments were only conducted if the total percentage of aggregation achieved ≥ 65 % following the addition of 8 µM of the agonist ADP (final concentration). AGE at the following final concentrations 1 %, 5 % and 10 % were added to pre-aggregated PRP in which aggregation was initiated with 8 µM ADP. These experiments were repeated using 8 µM Sin-1, 10 nM PGE_1.
- Following the addition of ADP, each reaction was allowed to run for a total of 8 - 10 mins to allow any disggregation to occur. The disaggregatory effects of all reagents were measured by calculating the reduction in total percentage aggregation compared to the control, (Naimushin and Mazurov 2003), (Fig. 2.1).

$$\left(\frac{\text{(Control Platelets – Treated Platelets)}}{\text{Control Platelets}}\right) \text{X } 100 = \text{Disaggregation Effect}$$

Fig.2.1- Formula to calculate the percentage of disaggregation of platelets.

2.7.2 Binding to immobilised fibrinogen

The effect of AGE and specific inhibitors, including NO donors upon fibrinogen binding were investigated using a simple adhesion assay. During platelet aggregation, an important process is the adhesion of one platelet to another allowing an aggregate to form, the process involves the binding of fibrinogen to its receptor GPIIb/IIIa and occurs in response to all stimulating platelet agonists. This process can be observed, with specific reference to NO and AGE treated platelets using a simple colourmetric assay. Platelets that have adhered to immobilised fibrinogen are stained using Rose Bengal solution. The adhered platelets are lysed, with the released Rose Bengal providing a measure of active platelet adherence to fibrinogen when quantified spectroscopically. The adherence of ADP activated platelets to immobilized fibrinogen can also be observed using the same assay, with the effect of NO and AGE upon fibrinogen binding also investigated.

To investigate the effects of AGE upon fibrinogen binding, a 96 well microtitre plate was coated with 3g/L of fibrinogen reconstituted in $_{d}H_2O$ and left overnight at 4 °C. Excess fibrinogen was removed and non-specific binding sites were blocked with the addition of 1 % BSA in PBS buffer (10 mM phosphate, 150 mM sodium chloride, pH 7.4), for 1 hour at room temperature. PRP was incubated with either PBS, 1 µM GR 144053 (final concentration), 8 µM Sin-1 (final concentration), various concentrations of AGE from 1-10 % (v/v) or Sin-1 and AGE (1 % or 10 %) for 10 minutes at 37 °C before being placed onto ice to stop the reaction. The platelets were then washed as described (2.4.2) in HEPES Tyrode buffer (138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 1mg/ml glucose, 20 mM HEPES, pH 7.4), twice for 5 mins at 1500 x g to remove any residual colour present from AGE.

Samples were added to respective wells on the fibrinogen coated 96 well plate. Platelets were either left inactive to measure spontaneous binding or activated by 8 µM ADP (final concentration). After 5 mins, the reaction was stopped by placement onto ice. Non-adherent cells were removed via aspiration, before washing the plate 3 times in PBS. 0.2 % (w/v) Rose Bengal solution was added to each well and left for 30 mins at room temperature. Excess Rose Bengal was removed via aspiration and the plate washed 3 times with PBS. Adherent platelets were lysed by the addition of lysis

buffer (1 % triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, 13 mM Sodium pyrophosphate, 50 mM NaF, 1.1 mM sodium orthovanodate, 10 % glycerol, 1 mM PMSF, 20 µg/ml apoprotein, 20 µg/ml leupeptin, 20 µg/ml pepstatin A, pH 5.6). The released Rose Bengal from the lysed platelets was measured on a microtitre plate reader at 540 nm (spectramax 190 plate reader, softmax pro v5). Various controls were tested on the 96 wells plate, including blank wells, fibrinogen only coated wells, rose Bengal with fibrinogen only, Rose Bengal only and platelets with fibrinogen only. These were investigated to ensure the released Rose Bengal from lysed platelets depicted an accurate result with no undue influence from any other reagent.

2.8 Measurement nitrite, a marker of nitric oxide

2.8.1 Griess assay

NO activity *in vivo* can be indirectly determined through the measurement of specific metabolites such as nitrite and nitrate. To assess NO signalling and whether AGE can affect NO activity, total nitrite concentration was measured. The final products of NO *in vivo* are nitrate and nitrite and total nitrite concentrations can be measured using a simple assay, the Griess assay. The assay involves any nitrite present forming a diazonium salt upon contact with sulphanilic acid. Upon further addition of an azo dye (alpha-naphthylamine) a pink color develops and can be read at 540nM (Fig. 2.2). The Griess assay has a detection limit of 3 μM nitrite, therefore lower concentrations may not be accurately determined. The Griess assay was utilised to observe the effect of AGE upon platelet produced NO and the effect of AGE upon NO generated from Sin-1.

To measure nitrite concentrations, platelets were washed as described (2.4.2) in HEPES Tyrodes buffer (138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 1 mg/ml glucose, 20 mM HEPES, pH 7.4). Platelets were then incubated with PBS (10 mM phosphate, 150 mM sodium chloride, pH 7.4), 8 μM Sin-1 (final concentration), 10 % (v/v) AGE or a prepared diethyl ether extract of AGE (DEA) (final concentration), both Sin-1 and AGE or DEA for 10 minutes at 37 °C before being activated with 8 μM ADP (final concentration) or directly placed onto ice to stop the reaction. Platelets were lysed by the addition of lysis buffer (1 % triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.2), 5 mM EDTA, 13 mM Sodium pyrophosphate, 50 mM NaF, 1.1 mM sodium orthovanodate, 10 % glycerol, 1 mM PMSF, 20 μg/ml apoprotein, 20 μg/ml leupeptin, 20 μg/ml pepstatin A, pH 5.6) and agitated before placing on ice for 5-10 minutes. The samples were then loaded onto a 96 well microtitre plate. A standard curve was constructed from 1.56 to 100 μM of sodium nitrite and also loaded onto the 96 well plate. The Griess reagent was made fresh as required and consisted of a 1:1 ratio of 1 % (w/v) sulfanilamide in 5 % phosphoric acid (v/v), to 0.1% (w/v) Naphtylethylene diaminedigydrochloride (NED) in $_dH2O$. Once equilibrated to RT, the Griess reagent was added to each sample on the plate inclusive of the standard curve. The plate was incubated at RT for at least 20 mins protected from light until a purple/magenta colour

formed. The absorbance was measured on plate reader at 540nm (spectramax 190 plate reader, softmax pro v5) within 30 minutes. Unknown platelet nitrite concentrations were calculated from the generated standard curve (Appendix IV).

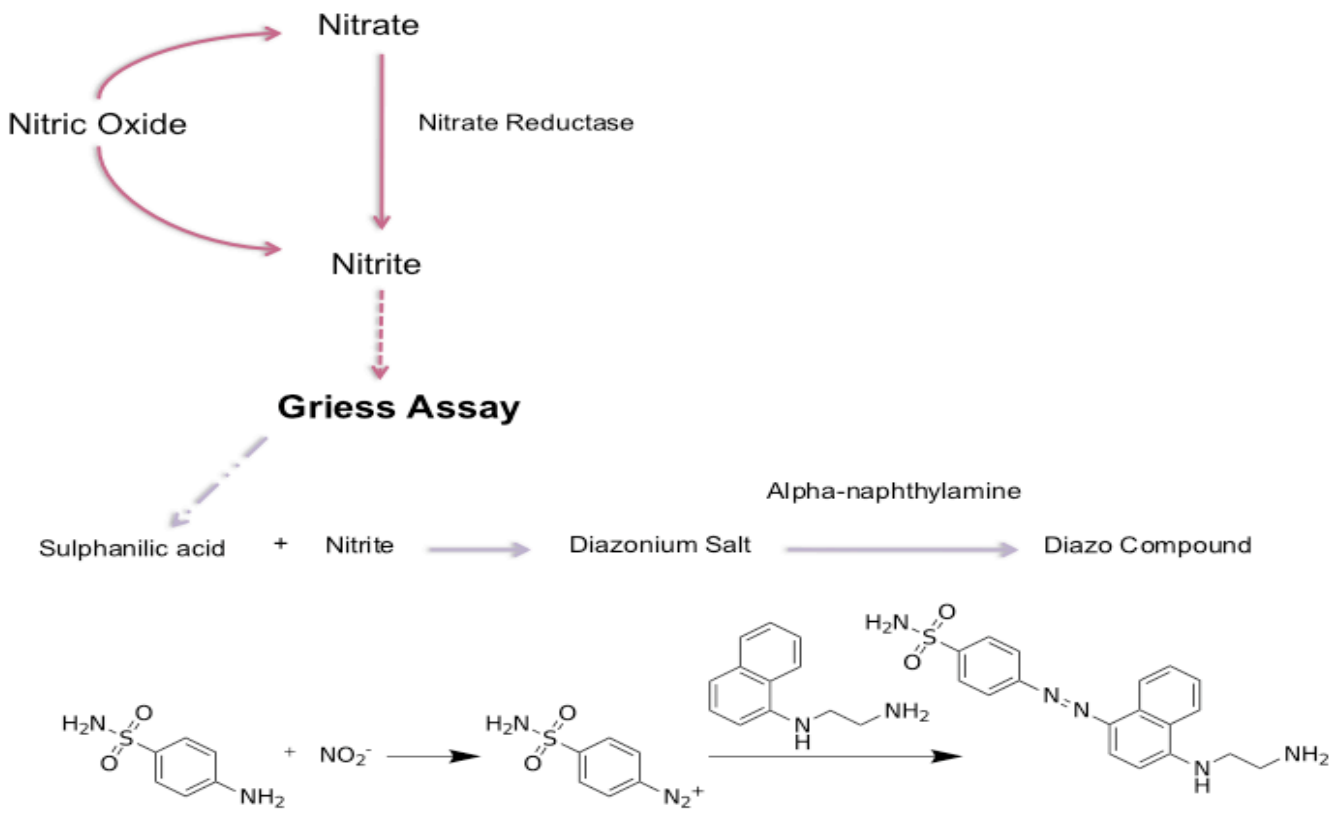


Fig 2.2:- Simplified schematic of the principle reaction of the Griess assay. Nitrite can be measured using a diazo compound, resulting in a colour change that can be read on a spectrophotometer at 540nm.

2.8.2 Nitrite detection using fluorescence

To detect lower concentrations of nitrite to 0.2 µM, a commercially available fluorescent Nitric Oxide kit was utilized (Calbiochem, Merck Biosciences). The assay is based upon the addition of 2,3-diaminonapthalene (DAN) and NaOH which coverts nitrite present in the sample into a fluorescent emitting compound. This fluorometric NO assay was utilized to observe the effect of AGE upon platelet produced NO, by using nitrite as a marker of NO activity the effect of AGE upon NO released from Sin-1 was also observed.

To prepare platelet samples for this assay, platelets were washed as previously described (2.4.2) with HEPES Tyrodes buffer (138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 1 mg/ml glucose, 20 mM HEPES, pH 7.4) and then incubated with PBS (10 mM phosphate, 150 mM sodium chloride, pH 7.4), 8 µM Sin-1 (final concentration), 10 % (v/v) AGE or DEA (final concentration) or both Sin-1 and AGE or DEA for 10 minutes at 37°C before being activated with 8 µM ADP (final concentration) or directly placed onto ice to stop the reaction.

Platelets were lysed by the addition of a Triton X-100 buffer that included protease inhibitors (1 % triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, 13 mM Sodium pyrophosphate, 50 mM NaF, 1.1 mM sodium orthovanodate, 10 % glycerol, 1 mM PMSF, 20 µg/ml apoprotein, 20 µg/ml leupeptin, 20 µg/ml pepstatin A, pH 5.6) and agitated before placing on ice for 5-10 minutes. The samples were then loaded onto a white 96 well plate provided with nitric oxide assay fluorometric kit (Calbiochem, Merck Biosciences, Nottingham, UK) and the manufacturers protocol followed. Briefly, a nitrite standard curve was prepared using standards provided. 2,3-diaminonapthalene (DAN) was added to each well and left for 10 min before stopping the reaction with 2.8 M NaOH. The plate was read using a fluorimeter (Varian Cary Eclipse), with an excitation wavelength of 365nm and an emission wavelength of 450nm. Unknown nitrite concentrations were extrapolated from a standard curve that was generated using nitrite standards of known concentrations (Appendix V)

2.9 Detection of nitrotyrosine

Nitrotyrosine is formed by the nitration of tyrosine residues. It is mediated by reactive nitrogen species and can serve as a marker of NO activity *in vivo*. Expression of nitrotyrosine can indicate NO dependent activity through RNS induced stress. Experiments were carried out using a specific antibody to observe whether platelet nitrotyrosine expression was altered after treatment with AGE, providing an indication whether AGE can influence NO activity.

2.9.1 Extraction of platelet proteins

Platelets washed as previously described (2.4.2) in HEPES Tyrodes buffer (138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 1mg/ml glucose, 20 mM HEPES, pH 7.4) and were centrifuged to pellet the platelets. A count of approximately 1 x 10^7 - 1 x 10^8 cells were added to 1 ml of lysis buffer (1 % triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, 13 mM Sodium pyrophosphate, 50 mM NaF, 1.1 mM sodium orthovanadate, 10 % glycerol, 1 mM PMSF, 20 µg/ml apoprotein, 20 µg/ml leupeptin, 20 µg/ml pepstatin A, pH 5.6) and agitated before placing on ice for 5-10 minutes. The lysate was stored at -20°C until use.

2.9.2 Determining protein concentration using Bradford assay

Unknown protein concentrations were established using Bradford assay. Several standards to make a standard curve ranging from 5 to 50 µg/µl were prepared using a working solution of BSA (10 mg/ml). The standards were prepared by adding 0.5 µl - 5 µl of 10 µg/µl BSA to 1 ml of Bradford reagent, resulting in final concentrations from 5 – 50 µg/µl of BSA. The colour change was read on a spectrophotometer at 595nm. Unknown protein samples, 5 µl, were added to 1 ml of Bradford reagent and the resulting optical densities were read from a standard curve allowing unknown protein concentrations to be extrapolated from the standard curve (Appendix VI).

2.9.3 Protein separation using SDS-PAGE

Platelet lysate extracts were either untreated or treated with 8 µM Sin-1, 10 % AGE (v/v) or both 8 µM Sin-1 and 10 % AGE (v/v) at 37°C for 10 minutes before being added to 2 x sample buffer (water, stacking gel buffer, glycerol, 10 % SDS (w/v) and 0.05 % bromophenol (w/v) blue with 4 % mercaptoethanol (v/v) for reduced samples). Samples were either heated at 95°C for 10 minutes to complete denaturing of the proteins or not heated and left as native proteins. Platelet protein samples of 40 µg/µl were loaded onto 2 separate 15 % polyacrylamide gels and ran at 50 V overnight with HyperPAGE prestained protein markers (Bioline, London, UK) used as a standard. One SDS-PAGE gel was stained with comassie brilliant blue and imaged using a Biorad gel doc and quantity one software. The second SDS-PAGE gel containing the same lysate samples was transfered onto polyvinylidene difluoride (PVDF) membrane for protein detection using specific antibodies.

2.9.4 Detection of nitrotyrosine via western blot

Resolved protein bands were transferred electrophorectically onto a PVDF membrane overnight at a constant rate of 30 V, using a transfer buffer of 192 mM glycine, 25 mM tris and 10 % methanol with pH adjusted to between 8 – 8.3. The successful transfer of all of the standards used in prestained HyperPAGE marker (Bioline, London, UK) indicated that proteins were successfully transferred from the SDS-PAGE gel to membrane. Non-antigenic sites on the membrane were blocked using 5 % non-fat dried milk (w/v) to reduce non-specific binding by the chosen antibody. This was followed by three washes in TN-TBS buffer (20 mM Tris, 100 mM NaCl, 1 ml tween 20, at pH 7.2) followed by incubation with the primary antibody IgG mouse monoclonal anti-nitrotyrosine at a concentration of 1:1000 and left overnight at 4°C. Excess antibody was removed by three washes in TN-TBS before being incubated with the secondary anti-mouse antibody, which was conjugated to horse-radish peroxidase (HRP). The incubation was carried out at room temperature for approximately 45 mins at a dilution of 1:20,000. The blot was then washed another three times in TN-TBS buffer before the addition of enzyme substrate Luminol (Santa Cruz Biotechnology, CA) and further development on ECL film (Amersham, UK) or imaged on a BioRad ChemiDoc through the chemiluminescence mode.

The housekeeping proteins actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) should remain consistent and were therefore utilized as loading controls to ensure equal protein concentrations across each sample. To identify the concentrations of the housekeeping proteins, the membrane was stripped via soaking in stripping buffer (0.2 M glycine, 3.5 mM SDS, 1% Tween-20 (v/v), pH 2.2), for 45 minutes followed by a wash thoroughly in TN-TBS and finally re-blocked in 5% non-fat dried milk (w/v). The primary antibody rabbit monoclonal for either GAPDH or Actin (Cell Signaling Technologies, UK) was used at 1:1000 and left overnight at 4°C. The membrane was further washed before the addition of a secondary HRP conjugated goat anti-rabbit antibody at 1:10,000 for 45 mins at RT. Excess antibody was removed via thorough washing with TN-TBS before further development with ECL reagents or imaged using chemiluminscence on a BioRad ChemiDoc.

2.10 Imaging of platelets

2.10.1 Scanning electron microscope imaging of platelets

Platelets ($1x10^8$) were first washed as previously detailed (2.4.2) in HEPES Tyrodes buffer (138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 1mg/ml glucose, 20 mM HEPES, pH 7.4) and were treated with PBS (10 mM phosphate, 150 mM sodium chloride, pH 7.4), 8 µM Sin-1 (final concentration), 10 nM PGE_1 (final concentration) or 5 % AGE (v/v, final concentration) at 37°C for 10 mins before the reaction was stopped by placement onto ice. Each suspension was further washed in HEPES tyrodes buffer to remove any residual colour and re-suspended into 500 µl of HEPES tyrodes buffer. Washed cells were then activated with 8 µM ADP (final concentration) and 100 µl aliquots of cells were taken at the specific time points, 0, 10, 30 and 60 seconds and placed directly into 100 µl of 2.5% (v/v) unbuffered glutaraldehyde and fixed overnight. Platelets were washed using PBS before being added onto a standard 13 mm aluminium SEM specimen stub and left to air-dry. Once dry, the samples were coated using EMITECH k550x gold sputter coater for 2.5 mins at 25 mA and Images were obtained using an Inspect S (FEI) Scanning Electron Microscope at 12.5 kV.

2.10.2 Fluorescent imaging of platelet actin

Platelets ($1x10^8$) were washed as detailed (2.4.2) with HEPES tyrodes buffer (138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 1mg/ml glucose, 20 mM HEPES, pH 7.4) followed by treatment with either PBS (10 mM phosphate, 150 mM sodium chloride, pH 7.4), 8 µM Sin-1 (final concentration), 10 nM PGE_1 (final concentration) or 5 % AGE (v/v, final concentration) at 37°C for 10 mins before the reaction was stopped by placement onto ice. Each suspension was further washed with HEPES tyrode buffer to remove any residual colour and re-suspended back into HEPES tyrodes buffer. Washed cells were activated with 8 µM ADP (final concentration) with an aliquot of cells taken at specific time points, 0, 10, 30 and 60 seconds respectively and placed directly into 2 % of the fixative paraformaldehyde. Platelets were briefly washed before being permeabilised with 0.1 % Triton X-100. Monoclonal mouse anti-actin

antibody conjugated to FITC (Sigma Aldrich) was added at a dilution of 1:50 diluted in PBS with 1 % BSA and left to incubate in the dark at room temperature for 1 hour. Each slide was carefully and thoroughly washed with PBS to ensure no residual antibody remained and left to air dry. Once sufficiently dry, one drop of anti-fade mounting media (Invitrogen) was added and cover slip placed onto each slide. Slides were kept in the dark until imaged using either an Olympus fluorescence microscope with Openlab software (4.0.4) or on a Zeiss 510, Laser Scanning Confocal microscope.

2.11 Statistical analysis

Experimental results are expressed as the means ± SEM and are accompanied by the number of observations (n). Data was first analysed to test for normal distribution using histogram analysis and linear regression when necessary. No data needed to be transformed before further analysis. Data was analysed using either a student's paired t test or oneway ANOVA (Microsoft excel toolpak statistical software add-on or SPSS v 15). T tests were conducted on data between two values with a minimum sample number of $n \geq 3$. Oneway ANOVA's were used where more than two means where being compared and had a minimum sample number of $n \geq 6$. A P value of less than 0.05 ($p \leq 0.05$) in any statistical analysis was considered statistically significant.

3 Reactive nitrogen species and their effects upon human platelets *in vitro*

3.1 Introduction

The agonist ADP initiates platelet activation resulting in complex cell signalling and the restructure of the actin cytoskeleton by binding to purinergic receptors on the platelet surface. The effect of ADP upon platelets is attributed to three P2 receptors, namely $P2Y_1$, $P2Y_{12}$ and $P2X_1$ (Erlinge and Burnstock 2008, Dorsam and Kunapuli 2004, Jin *et al* 1998). The activation of platelets mediated by ADP requires simultaneous stimulation of two of these purinergic receptors, $P2Y_1$ and $P2Y_{12.}$

The mobilisation of intraplatelet Ca^{2+} is essential to mediate ADP induced shape change. Activation by ADP will cause a decrease in the intraplatelet secondary signalling molecules cAMP and cGMP. The decrease in cAMP is mediated through inhibition of AC by the binding of ADP, as during ADP induced aggregation, adenylyl cyclase that is coupled to $P2Y_{12}$, is inhibited. Upon ADP binding to the receptor $P2Y_{12}$ the pertusis toxin (Gα) inhibits AC preventing the catalysis of ATP to cAMP, prohibiting further platelet signalling by cAMP (Hardy *et al* 2004). Therefore inhibition of AC with a chemical inhibitor such as SQ 22536 should promote an aggregatory response, surpassing the normal ADP stimulatory response, which naturally inhibits AC (Haslam *et al* 1978, Daniel *et al* 1999, Cho *et al* 2007).

An increase in platelet cGMP occurs through the direct stimulation of sGC by NO and potently inhibits platelet aggregation (Naseem and Roberts 2010). The intracellular receptor sGC is a heterodimeric protein with a haem binding subunit (Zhang *et al* 2011). There is evidence that sGC has a contradictory biphasic role within platelets, as the cytosolic enzyme can both partake in the activation of platelets and mediate NO induced inhibition of platelets (Zhang *et al* 2011). Using the non-enzymatic spontaneous NO donors, 3-Morpholinosydnonimine hydrochloride (Sin-1) and Sodium nitroprusside (SNP), cGMP can be increased via sGC (Thomas *et al* 2008).

Elevation of either cyclic nucleotide promotes further signalling within platelets, including the binding of specific protein kinases that induces further signalling. Regulation of platelet cAMP and cGMP is mediated through specific phosphodiesterases (PDE),

namely PDE 2, PDE 3 and PDE 5. This regulation plays an important role as too little or too much can either inhibit necessary platelet activation or help to induce unwanted platelet activation. Inhibiting PDE with the inhibitors IBMX and Zaprinast will allow cGMP and cAMP to accumulate resulting in a decrease in platelet aggregation.

3.2 Aims and objectives of the studies in this chapter

The aim of the investigations were to establish the role of NO and cyclic nucleotide signalling in platelet functions.

This was achieved using several different chemical mimetics and inhibitors. These inhibitors included the NO donors Sin-1, SNP, the inhibitors SQ 22536, ODQ, IBMX and Zaprinast and PGE_1. The use of these chemical mimetics allowed specific molecules in the signalling cascade to be isolated.

3.3 Overview of experimental approach

To investigate the involvement of cyclic nucleotides in platelet functions, the experimental procedures listed below were performed:-

- Aggregation studies investigating the effect of specific stimulators and inhibitors on ADP induced platelet aggregation (see section 2.5, p32)
- Enzyme linked immunoabsorbant assays investigating intraplatelet concentrations of both the cyclic nucleotides cAMP and cGMP (2.6, p37)
- Disaggregation of platelets by increasing intraplatelet cyclic nucleotide concentrations (2.7.1, p39)
- Fibrinogen Binding Assay to assess the effects of specific inhibitors on ADP induced platelet binding to fibrinogen (2.7.2, p40)
- Measurement of platelet NO metabolite nitrite (2.8, p42)
- SDS PAGE + Western Blot of platelet proteins highlighting the presence of nitrotyrosine in platelets after treatment with Sin-1 (2.9, p46)
- SEM imaging of the shape profile of ADP activated platelets after treatment with cyclic nucleotide increasing agents (2.10.1, p49)
- Fluorescent imaging of platelet actin structure in ADP activated platelets after treatment with cyclic nucleotide increasing agents (2.10.2, p49)

3.4 Results

3.4.1 The effect of NO on ADP induced platelet aggregation *in vitro*

3.4.1.1 *ADP induced aggregation*

The activation of platelets by ADP displays two phases of aggregation, a primary and a secondary phase. During the primary phase, higher rates of aggregation are observed due to the initiation of multiple signalling cascades. The secondary phase is much slower and is irreversible, as it is at this point that the platelet aggregate is stabilising through the secretion of further chemotactical agents including ADP and thromboxane A_2 (TXA_2) (Charo *et al* 1977, Zhou and Schmaier 2005, Packham and Rand 2011).

The average person will achieve approximately 90 % platelet aggregation after stimulation with ADP *in vitro*. Therefore only ADP induced aggregation curves that achieved a minimum of 65 % total percentage aggregation were deemed viable to be further analysed. Each aggregation curve was analysed for the primary and secondary rates of aggregation per second, as well as the total percentage of aggregation achieved over a maximum of 4 mins. The rates of aggregation were determined by identifying the start of each phase and calculating the percentage of aggregation per second for each ADP induced curve.

A dose dependence study was initially conducted to investigate the optimal concentration of ADP to induce full platelet aggregation. This preliminary study investigated concentrations from 1 µM to 400 µM and identified a final concentration of 8 µM to be optimal. At 8 µM a normal biphasic curve was observed that is characteristic of ADP induced platelet aggregation. This included an initial fast primary phase and slower secondary phase, with an average primary aggregation rate of 1.16 % / sec, whereas the average secondary aggregation rate was 0.24 % / sec. Further aggregation experiments will therefore utilise a concentration of 8 µM of ADP to induce platelet aggregation (Fig. 3.1).

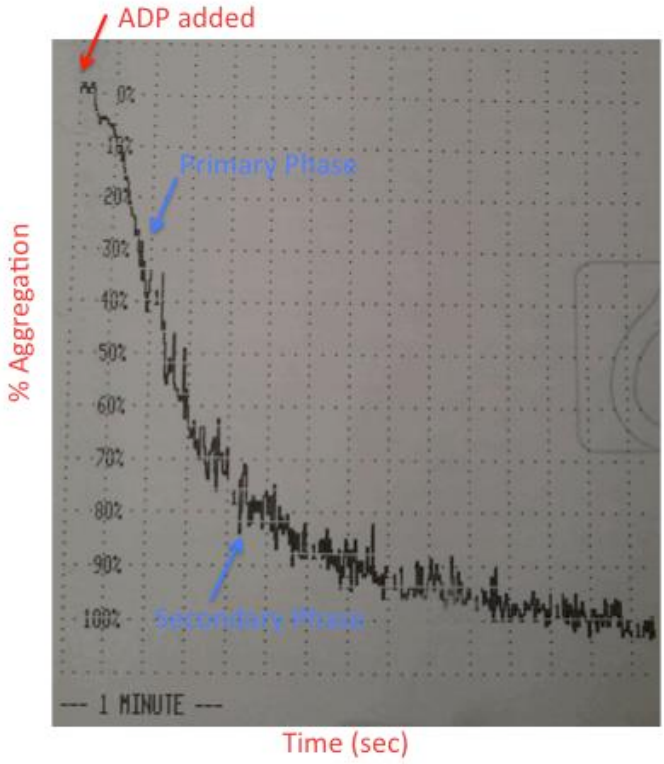


Fig. 3.1 – Aggregation curve of ADP induced aggregation. Control Aggregation plot showing aggregation of platelets when stimulated with the agonist ADP with the primary and secondary phases of aggregation indicated.

3.4.1.2 *The effect of NO donors on agonist induced platelet aggregation*

Platelets were treated with either Sin-1 or SNP, both of which produce NO, and will stimulate synthesis of cGMP therefore inhibiting platelet aggregation. Concentrations of each NO donor were determined from an initial dose dependent study. These studies indicated that the optimum concentrations for Sin-1 and SNP were 8 µM and 20 µM respectively. The incubation time period was also investigated, with 1 min at 37°C providing approximately 50 % inhibition of total percentage of aggregation for both Sin-1 and SNP. The incubation time and final concentrations which caused approximately half maximal inhibition (IC_{50}) were chosen for further experimentation (Fig. 3.2, Fig. 3.3).

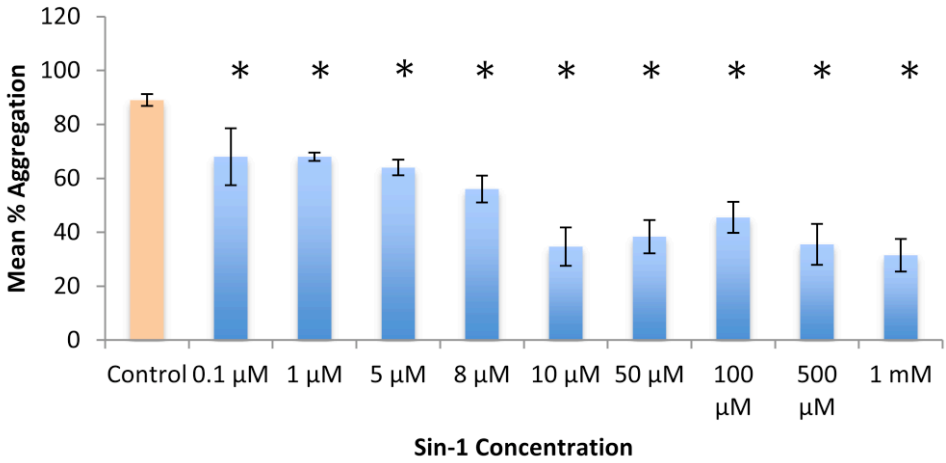


Fig. 3.2:- The effect of Sin-1 concentration on platelet aggregation

Determining the optimum concentration of Sin-1 for use in platelet aggregation investigations. Mean total percentage of ADP induced platelet aggregation ± SEM. Platelets were first treated with stated doses of Sin-1 (final concentrations) before aggregation was initiated with ADP. Statistical significant data is indiated by * compared to the ADP control, calculated using a paired students t test with p = 0.03 (n=6).

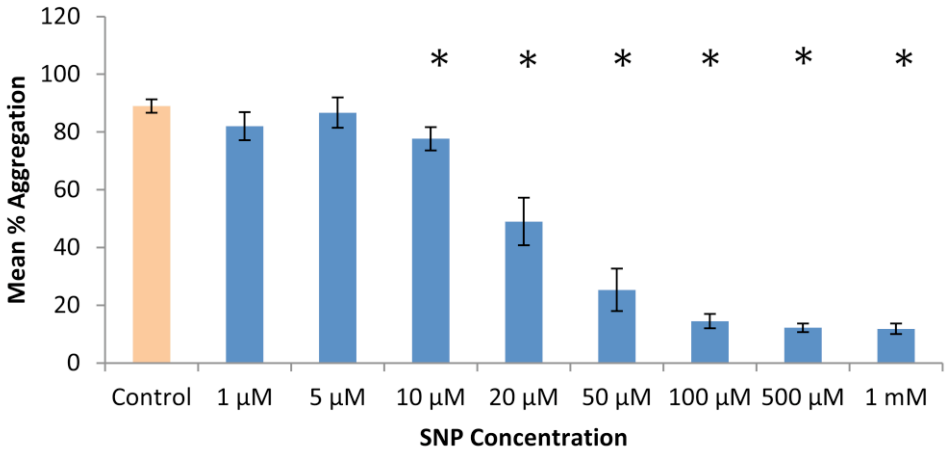


Fig. 3.3:- The optimum concentration of SNP for use in platelet aggregation investigations. Mean total percentage of ADP induced platelet aggregation ± SEM. Platelets were first treated with stated doses of SNP (final concentrations) before aggregation was initiated with ADP. Statistical significant data is indiated by * compared to the ADP control, calculated using a paired students t test with p = 0.02 (n=6).

PRP was treated with either 8 µM Sin-1 or 20 µM SNP (final concentrations) and aggregation was initiated by ADP. The addition of Sin-1 and SNP both caused inhibition of

platelet aggregation, with Sin-1 reducing aggregation to 62.5 % ± 4.04 % and SNP reducing aggregation to 65 % ± 4.2 % compared to the ADP control value of 97 % ± 1.2 % (Fig. 3.4).

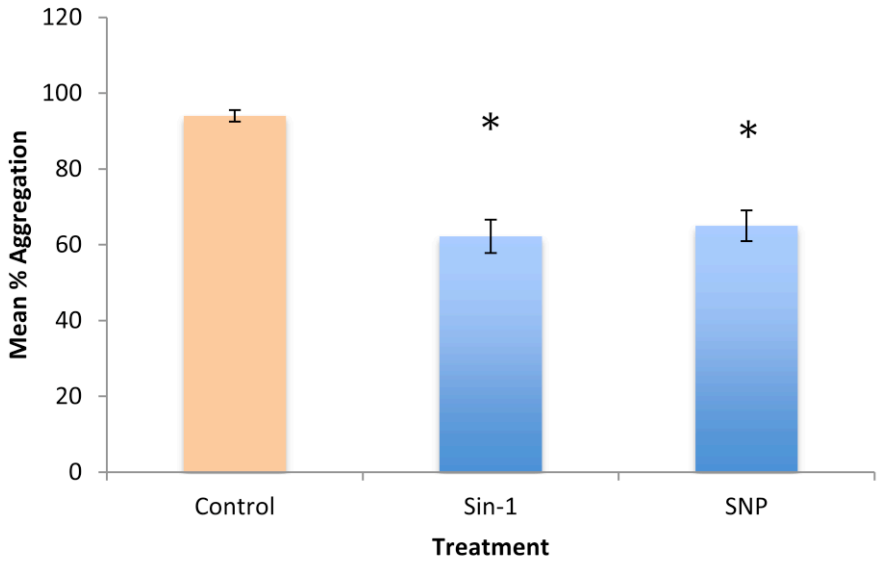


Fig. 3.4:- The effect of RNS on ADP induced platelet aggregation. Mean percentage of ADP initiated platelet aggregation with platelets treated with NO donor Sin-1 or SNP. Statistically significant points with p = 0.02 are indicated via * above respective point calculated via students paired t test to control data (orange) (n=6).

As Sin-1 produces both peroxynitrite and NO, it was necessary to establish that the generated $ONOO^-$ from Sin-1 had no effect upon platelet aggregation. Superoxide dismutase (SOD) is an enzyme that can catalyse present superoxides into oxygen and hydrogen peroxide (H_2O_2). The presence of SOD with Sin-1 will remove the generated $ONOO^-$ as well as other possible free radical species allowing the full effect of only NO by Sin-1 to be observed,

PRP was treated with SOD 10 units followed by further treatment with 8 µM Sin-1 (final concentration) before aggregation was initiated by ADP (Fig. 3.5). SOD alone had no effect upon platelet aggregation with a total percentage value of 96.3 % ± 1.88 % comparable to the control at 97 % ± 1.2 %. The combination of SOD and Sin-1 displayed no significant difference when compared to Sin-1 alone. The total percentage of aggregation achieved with SOD and Sin-1 of 54 % ± 5.7 % is within the error margins of Sin-1 alone

data of to 62.5 % ± 4.04 %. This indicates that the generated peroxynitrite from Sin-1 does not have an effect upon platelet aggregation (Fig. 3.5).

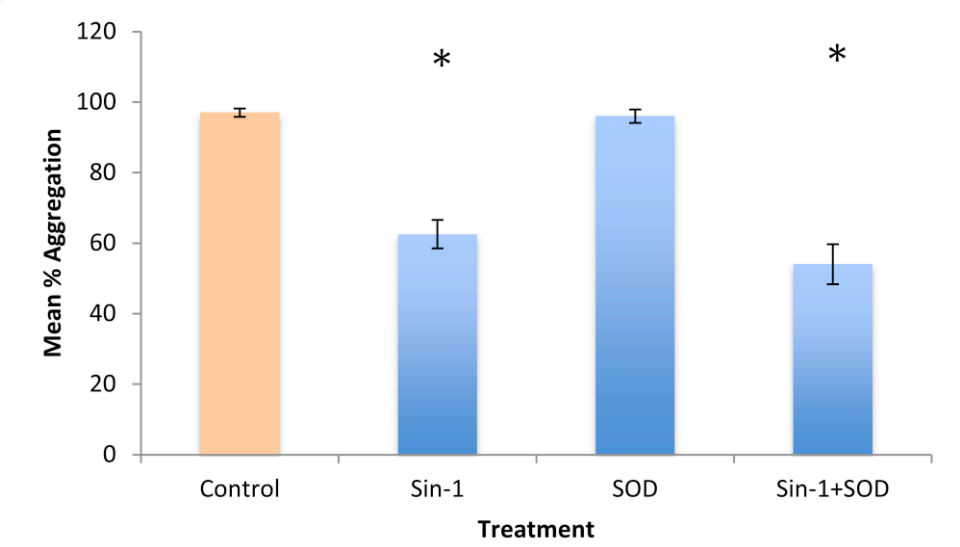


Fig. 3.5:- The effect of SOD and peroxynitrite on platelet aggregation. Mean percentage of ADP initiated platelet aggregation with platelets treated firstly with SOD before further incubation with Sin-1. Statistically significant points with p = 0.04 are indicated via * above respective point calculated via students paired t test to control data (orange). SOD data was not significant against the ADP control (n=6).

To determine the full effects of NO upon aggregation, the enzyme soluble guanylyl cyclase was investigated in combination with the NO donor Sin-1.

3.4.1.3 *The effect of inhibiting soluble guanylyl cyclase upon NO induced inhibition of platelet aggregation*

NO acts largely through influencing the generation of cGMP via sGC. Inhibiting sGC should remove any effect of Sin-1 and therefore NO upon platelet aggregation. The inhibitor 1*H*-[1,2,4]Oxadiazolo[4,3-α]quinoxalin-1-one (ODQ) selectively and irreverisibly binds to the haem site of sGC, blocking the binding of NO and therefore the transduction of NO signalling. The effect of inhibiting sGC and the effect on NO mediated inhibition of platelet aggregation was investigated.

The sGC inhibitor, ODQ was reconstituted in DMSO and further diluted using PBS. The vehicle control utilised an equal dilution of DMSO to ensure there was no effect. As DMSO can severaly impact on platelet functionality (Rosenblum and El-Sabban 1982), the concentration of the vehicle was consistently less than 0.5 % in all experiments.

PRP was first incubated with 100 µM ODQ (final concentration) before further treatment with 8 µM Sin-1 (final concentration). The incubation time of 10 mins proved to be suffcient to allow ODQ to permeate into the platelet. The concentration of 100 µM ODQ displayed no adverse effects upon platelets alone and has previously shown to be effective at preventing an NO induced increase in cGMP (Garthwaite *et al* 1995, Hwang *et al* 1998). Aggregation was initiated by the addition of 8 µM ADP (final concentration). (Fig. 3.6).

The diluted DMSO vehicle control displayed a minimal percent of inhibition of platelet aggregation of 92 % ± 2.55 %, compared to the control value of 97 % ± 1.2 %. Low concentrations of Sin-1 at 8 µM inhibited platelet aggregation by 35 %. A small decrease in platelet aggregation with ODQ was observed decreasing aggregation to 90 % ± 2.08 % from 92 % ± 2.55 % of the vehicle control. This percentage of inhibition caused by ODQ is similar to the vehicle control, therefore can be attributed to the presence of diluted DMSO which was less than 0.5 % (v/v). The inhibitory effects of 8 µM Sin-1 on platelet aggregation in the presence of ODQ were fully removed, with the total percentage aggregation back up to 94 % ± 6.34 % within error range of the control 97 % ± 1.2 %. This indicates that ODQ is able to reverse the effects of Sin-1 upon platelet aggregation (Fig. 3.6).

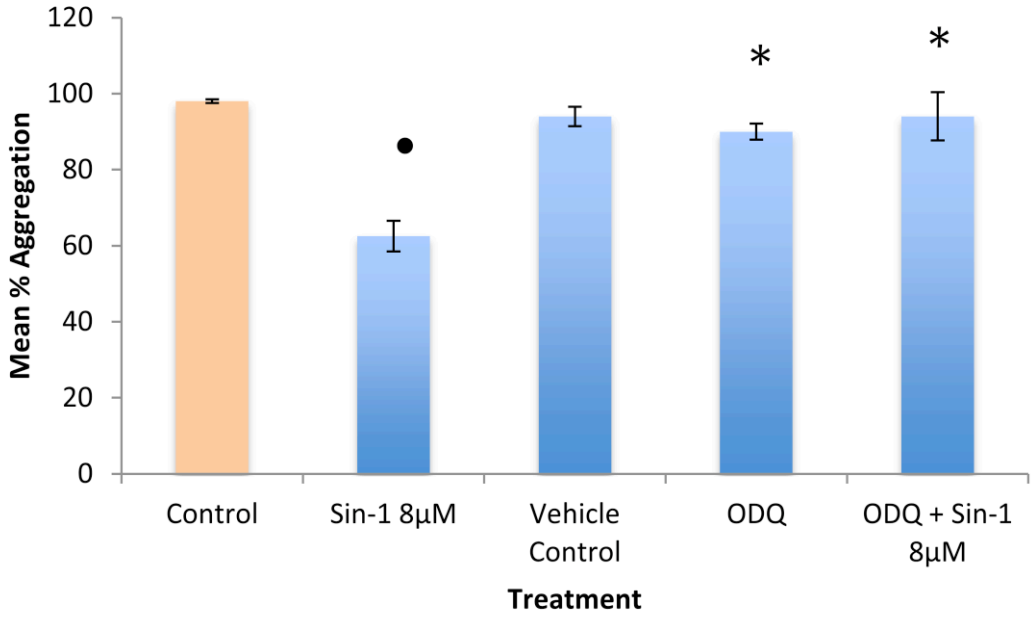


Fig.3.6:- The effect of 8 µM Sin-1 and ODQ upon platelet aggregation. Percentage platelet aggregation ± SEM after platelets were first incubated with ODQ before being challenged with a low dose of Sin-1 with ADP initiating aggregation. Statistically significant data was calculated to Sin-1 data via paired students t test, with p = 0.04 indicated by * above respective data point. • indicates significance to ADP control data p = 0.02 (orange). The vehicle control displayed no significant difference to the control (n=6).

To investigate whether during ADP induced platelet activation cGMP independent mechanisms are induced by NO, ADP induced platelet aggregation in the presence of ODQ was repeated using the higher concentration of 100 µM of Sin-1.

PRP was treated with 100 µM ODQ (final concentration) prior to the addition of 100 µM Sin-1 (final concentration) before aggregation was initiated by the addition of ADP. PRP was also treated with an appropriate amount of diluted DMSO, which is indicated as the vehicle control, and merginally reduced aggregation to 92 % ± 2.55 % from 97 % ± 1.2 % (Fig. 3.7).

Using a low concentration of Sin-1 the inhibition of platelet aggregation was demonstrated to be cGMP dependent, as the presence of ODQ removed the inhibitory effects of Sin-1 (Fig.3.3). The high concentration of 100 µM Sin-1 displayed an increase in inhibition of platelet aggregation by 67 % when compared to the inhibition of aggregation observed by 8 µM of Sin-1. In the presence of ODQ, the inhibition of aggregation induced

by 100 µM Sin-1 was not fully reversed, as aggregation was still decreased to 44.5 % ± 2.73 % from 90 % ± 2.08 % of ODQ alone (Fig. 3.7).

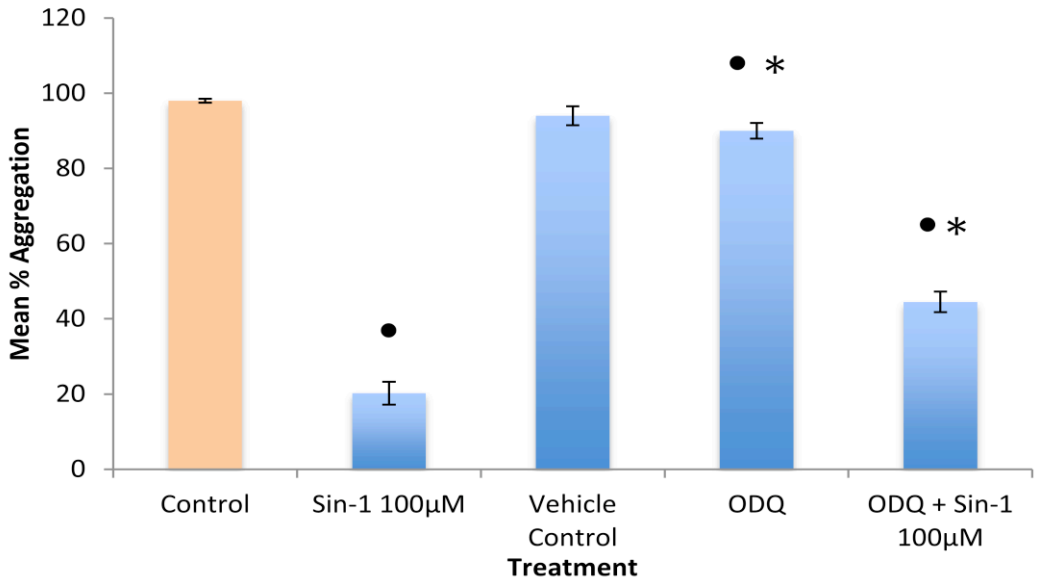


Fig 3.7.- The effect of 100 µM Sin-1 and ODQ upon platelet aggregation. Percentage platelet aggregation ± SEM, of platelets were first incubated with ODQ before being challenged with a high dosage of Sin-1, with aggregation initiated by ADP. Statistically significant data was calculated to Sin-1 data via students t test, with p = 0.04 is indicated by * above respective data point. • indicates significant data compared to control p = 0.01 (orange). The vehicle control was not significantly different to the ADP control (n=6).

As inhibiting sGC has a small inhibitory effect upon platelet aggregation, the effect of inhibiting Adenylyl cyclase, a key receptor for ADP induced platelet aggregation, was investigated.

3.4.1.4 *The effect of inhibiting adenylyl cyclase on platelet aggregation*

During ADP induced platelet aggregation adenylyl cyclase is inhibited, therefore the use of a chemical inhibitor should act to increase platelet aggregation (Fig. 3.8). Isolated platelets were incubated with 100 µM of SQ 22536 followed by treatment with 10 nM PGE_1 or PBS before ADP initiated activation.

Using the inhibitor of AC SQ 22536, a pro-aggregatory response by increasing aggregation to 113 % ± 0.8 % from 97 % ± 1.2 % of the ADP control in viable platelets. The

AC inhibitor SQ 22536 was reconstituted in $_dH_2O$, therefore there was no need for a vehicle control. PGE_1 stimulates the synthesis of cAMP through AC and was reconstituted in ethanol. To avoid any effect of ethanol on platelet function, PGE_1 once solubilised was further diluted using PBS with the final amount of ethanol present less than 0.5 % (v/v) in any given reaction. To ensure that this minimal amount of ethanol did not interfere with aggregation, a vehicle control utilizing the same diluted concentration of ethanol was also investigated. The vehicle control reduced aggregation to 89 %± 4.6 % from 97 %± 1.2 % of the ADP control.

Increases in cAMP will inhibit platelet aggregation, therefore using the stimulator of cAMP, PGE_1 inhibition of platelet aggregation should be observed. PGE_1 decreased the total percentage aggregation to 30.3 % ± 4.98 % from the vehicle control percentage of 89 %± 4.6 %. The inhibition of aggregation induced by PGE1 in the presence of SQ 22536 is removed, as aggregation is increased to 98 % ± 8.38 % from 30.3 % ± 4.98 % of PGE_1 alone. This indicates that SQ 22536 as expected is reversing the inhibitory effects that PGE_1 normally exerts upon platelet aggregation (Fig. 3.8).

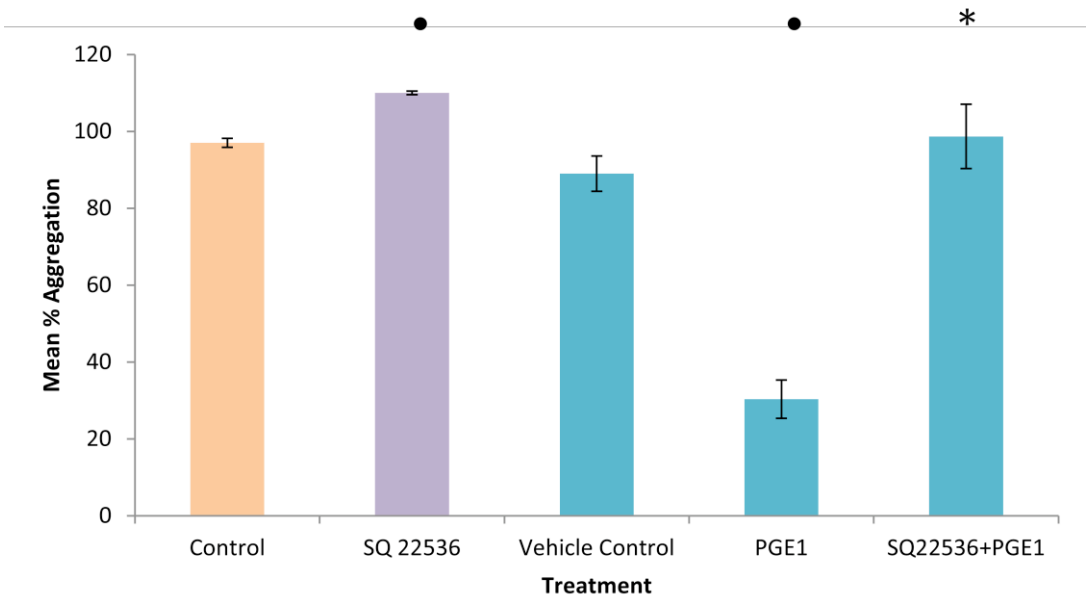


Fig 3.8:- The effect of inhibiting AC on platelet aggregation. Average percentage of ADP induced platelet aggregation ± SEM. Platelets were incubated with or without SQ22536 before further treatment with PGE_1. Aggregation was initiated by ADP and * Indicates statistical significant value p = 0.006 when conducting a paired t-test to the control data of PGE_1. Significant data compared to the control is indicated with • with p = 0.03 (n=6).

The addition of SQ 22536 caused an increase in aggregation, in contrast ODQ caused a small amount of inhibition of platelet aggregation. To investigate the effect of inhibiting both AC and sGC the chemical inhibitors SQ 22536 and ODQ were utilised. The inhibition of either sGC or AC should decrease intraplatelet cGMP and cAMP respectively.

3.4.1.5 *The effect of Inhibiting soluble guanylyl cyclase and adenylyl cyclase on platelet aggregation*

Using stimulators of sGC and AC, Sin-1 and PGE_1 respectively, each inhibitor successfully reversed the inhibitory effects that both Sin-1 and PGE_1 provoked, indicating that each respective inhibitor is functioning as expected. These experiments aim to both check the efficiency of the inhibitors and show that the inhibitors ODQ and SQ 22536 are fit for purpose. This will be achieved by challenging the platelet with stimulators of each respective enzyme after first treating with the inhibitors ODQ and SQ 22536 for sGC and AC. The inhibitory effects of Sin-1 and PGE_1 on platelet aggregation should be reversed if each inhibitor is efficiently working (Fig. 3.9).

The effect of inhibiting both sGC and AC simultaneously was investigated first. PRP was incubated with 100 μM ODQ and 100 μM SQ22536 (final concentration) before aggregation was induced by ADP. The presence of ODQ was comparable to the vehicle control with total percentage of aggregation 90 % ± 2.08% and 92 % ± 2.55 % respectively. Whereas SQ 22536 increased platelet aggregation as expected to 113 % ± 0.8% from the ADP control of 97 % ± 1.2%. Inhibiting both enzymes sGC and AC with ODQ and SQ 22536 respectively caused more inhibition of aggregation than either inhibitor alone decreasing aggregation to 84 % ± 4.7 % (Fig. 3.9).

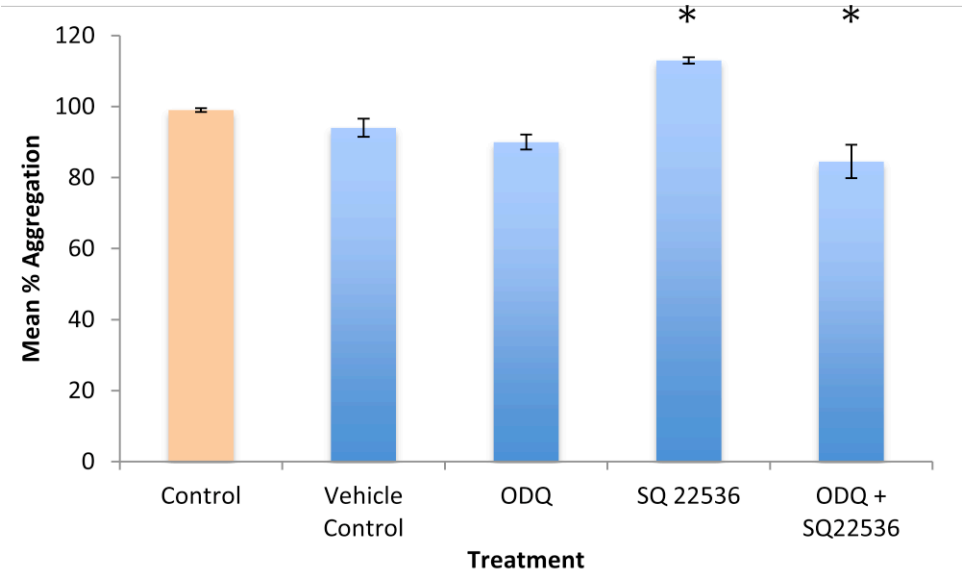


Fig 3.9:- The effect of inhibiting both sGC and AC upon platelet aggregation. Percentage platelet aggregation ± SEM. Platelets were incubated with one or both SQ22536 or ODQ before ADP initiated aggregation. Statistically significant data was calculated to the vehicle control data with p = 0.05 are indicated with * above respective point (n=6).

To test that each inhibitor was working correctly, platelets treated with ODQ and SQ 22536 were further challenged with the stimulators PGE_1 and Sin-1. Platelets were challenged with PGE_1 and Sin-1 or SQ 22536, ODQ, PGE_1 and Sin-1 (Fig. 3.10).

The stimulators Sin-1 and PGE_1 for cGMP and cAMP respectively, significantly inhibited platelet aggregation when combined decreasing aggregation to 12 % ± 5.7 % from 9 % ± 1.2 % of the ADP control. The presence of ODQ and SQ 22536, however, reversed this inhibition of aggregation by Sin-1 and PGE_1 increasing aggregation to 81 % ± 5.6 %, which is comparable to the percentage of aggregation that the inhibitors alone achieve of 84 % ± 4.7 % (Fig. 3.10). These results show that the inhibitors are successfully able to reverse the inhibitory effects of Sin-1 and PGE_1.

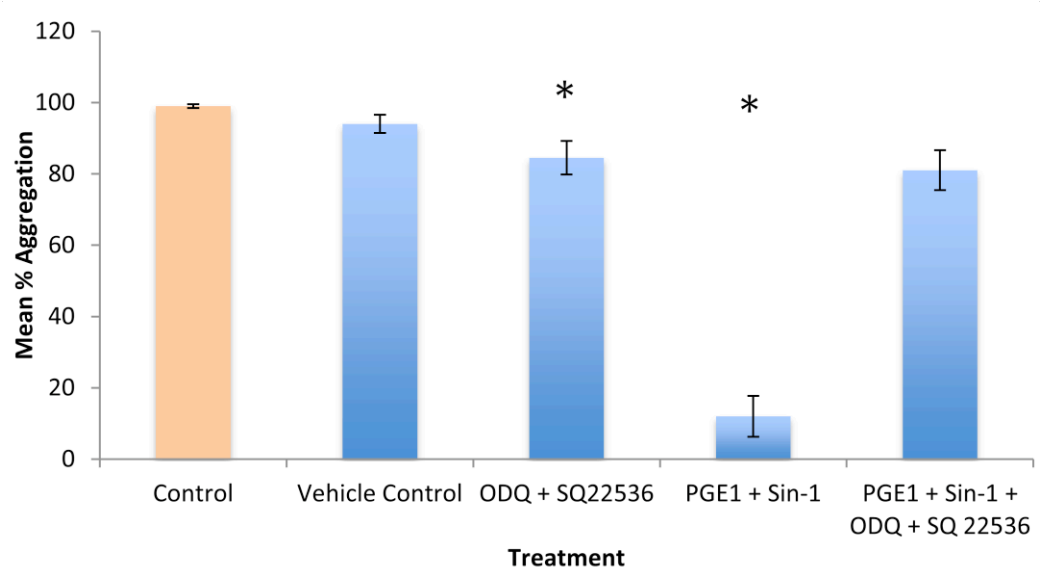


Fig 3.10:- The effect of inhibiting both sGC and AC on Sin-1 and PGE_1 treated platelets. Percentage platelet aggregation ± SEM. Platelets were incubated with one or both SQ22536 or ODQ before further treatment with Sin-1, PGE_1 or both. Aggregation was initiated by the addition of ADP. Statistically significant data was calculated to the vehicle control data with p = 0.038 are indicated with * above respective point (n=6).

To fully investigate the effects of either inhibitor on aggregation, each aggregation plot was analysed for the primary and secondary aggregation rates that are characteristic of ADP induced platelet aggregation.

The primary rate of aggregation was increased in the presence of the AC inhibitor, SQ 22536 to 1.4 % / sec from the ADP control rate of 1.16 % / sec. ODQ alone exhibited a decrease in the primary rate to 0.93 % / sec, however in the presence of both inhibitors SQ 22536 and ODQ the rate increased to 1.33 % / sec. Secondary rates of ADP induced platelet aggregation displayed little difference with values between 0.23 % / sec – 0.25 % / sec, except for SQ 22536 which decreased the rate of aggregation to 0.16 % / sec exhibiting the slowest rate (Table 3.1).

Inhibitor	Primary	SEM	Secondary	SEM
	% / sec		% / sec	
Control	1.16	0.04	0.24	0.07
SQ 22536	1.42	0.11	0.16	0.02
ODQ	0.93	0.07	0.23	0.6
SQ 22536 + ODQ	1.33	0.1	0.25	0.12
P Value	0.002 *		0.01 *	

Table 3.1:- The effect of inhibitors on the rates of ADP induced aggregation. Average rates of ADP induced platelet aggregation in the presence of sGC and AC inhibitors ODQ and SQ 22536 respectively. P values were calculated using an ANOVA for each of the primary rate and secondary rate data (n=6).

Statistical analysis using an ANOVA was performed on the primary and secondary rates of aggregation across each inhibitor. Both the primary and secondary rates were shown to be statistically significant with $p \leq 0.01$. This indicates that each inhibitor has a significant influence upon platelet aggregation.

Inhibiting AC and sGC will result in a decrease in platelet cyclic nucleotides whose concentrations are regulated by platelet PDE.

3.4.1.6 *The effect of inhibiting platelet phosphodiesterases*

The purpose of these experiments was to highlight the role of PDE upon cyclic nucleotide stimulation and degradation. Examining the effects of PDE in platelet functions will allow further dissection of the signalling pathways that cyclic nucleotides are involved in within platelet biochemistry. As Sin-1 increases platelet cGMP, inhibiting PDE using IBMX should potentiate the inhibition of aggregation caused by Sin-1 alone.

IBMX is a non-selective PDE inhibitor and can have other effects upon the cell including influencing PKC activity as well as the inhibition of PDE (Williams *et al* 1987, Essayan 2001). Despite these non-specific effects of IBMX, the inhibitor was chosen, as it should inhibit all present isoforms of platelet PDE and is widely used as a PDE inhibitor. PRP was pre-treated with 100 µM IBMX (final concentration) for 30 min to allow IBMX to fully permeate the platelets. Platelets were further treated with Sin-1 and ADP initiated aggregation.

When added to PRP, IBMX caused a significant decrease in aggregation to 63.2 % ± 2.69 % from 97 % ± 1.2 % of the ADP control. Treatment with the NO donor Sin-1 caused a similar amount of inhibition to IBMX alone decreasing aggregation to 62 % ± 4 %. The effect of combining IBMX and Sin-1 is highly significant to both IBMX alone data and Sin-1 alone data as in the presence of IBMX, Sin-1 significantly decreases aggregation to 10 % ± 2 %. These observations indicate the potent inhibitory effects caused by cyclic nucleotides upon ADP induced platelet aggregation. The vehicle control displays the effect of diluted DMSO in which IBMX is solubulised displaying a small decrease in aggregation to 92 % ± 2.55 % from 97 % ± 1.2 % of the control (Fig. 3.11).

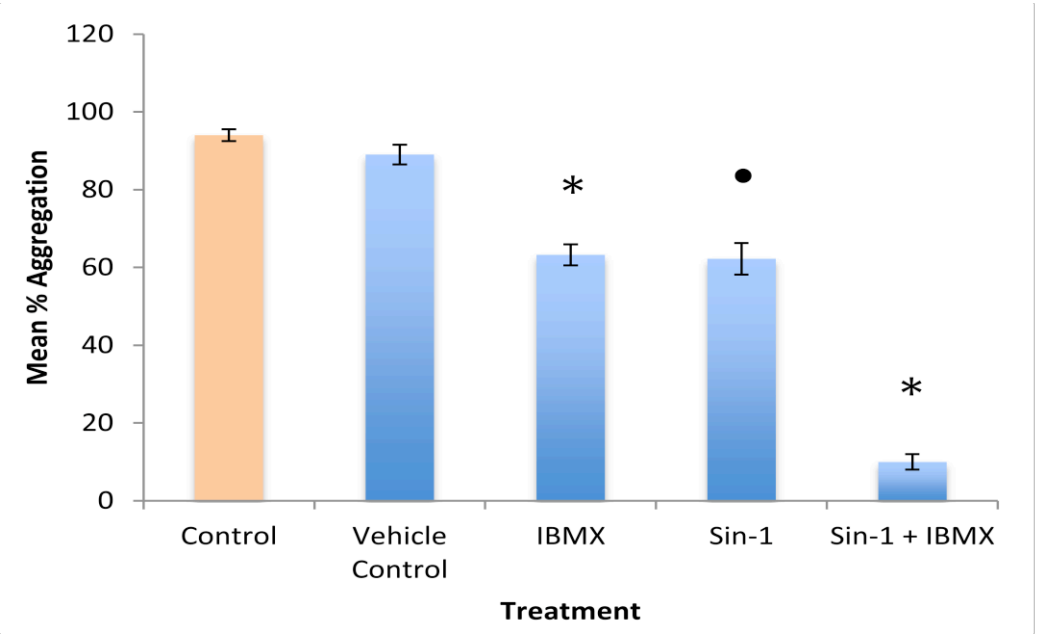


Fig. 3.11:- The effect of IBMX on NO mediated inhibition of platelet aggregation. The average percentag of platelet aggregation ± SEM after platelets were incubated with IBMX before ADP initiated aggregation. Statistically significant points were calculated using students T test between respective controls. Significant data are marked with * above the respective point show significance to vehicle control p = 0.018. • indicates significance to control data (orange) with p = 0.029 (n=5).

For a more specific approach to investigate the effect of cGMP upon aggregation, the inhibitor Zaprinast was utilised. Zaprinast is a selective inhibitor of cGMP specific PDE5 within platelets and should cause a decrease in the aggregation of platelets, due to the accumulation of intraplatelet cGMP. These experiments differ to those conducted with

IBMX as Zaprinast is specific for PDE5, which will allow the accumulation of only cGMP, contrasting to IBMX which will allow both cAMP and cGMP to accumulate. The combination of Sin-1 and Zaprinast should significantly increase the inhibition of platelet aggregation when compared to Sin-1 alone.

Platelets were incubated with 0.76 µM Zaprinast (final concentration or with an appropriate amount of diluted DMSO less than 0.5 % (v/v), in which Zaprinast was reconstituted. For tests investigating Zaprinast and Sin-1, PRP was further incubated with Sin-1 with aggregation initiated by the addition ADP.

Zaprinast alone shows a small decrease in ADP induced platelet aggregation decreasing to 86 % ± 3.05 % from the vehicle control value of 92 % ± 2.55 %. In the presence of Zaprinast and Sin-1, a significant decrease in aggregation is observed decreasing aggregation to 21 % ± 4.87 %, from Zaprinast alone at 86 % ± 3.05 %. These results indicate the potent inhibitory effect of accumulated cGMP upon platelets (Fig 3.12).

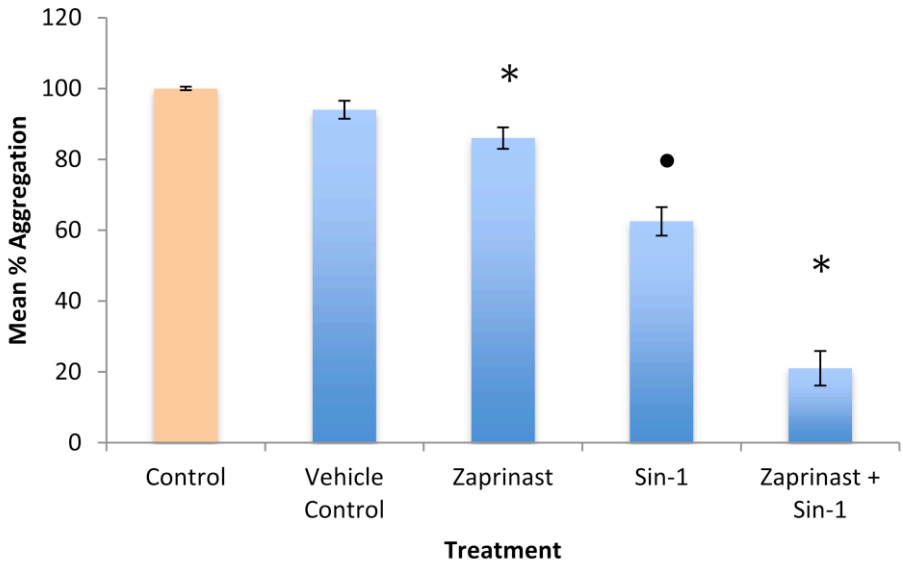


Fig. 3.12:- The effect of Zaprinast on NO mediated inhibition of platelet aggregation. Average percentage of platelet aggregation ± SEM when incubated with Zaprinast before aggregation was initiated with ADP. Statistically significant points were calculated using students T test between, respective control points. Significant data to the control are indicated by • with p = 0.02 Data marked with * are significant to the vehicle control with p = 0.04 (n=4).

3.4.2 Platelet cGMP

3.4.2.1 *The effect of Sin-1 and ODQ on intraplatelet cGMP*

Elevation of secondary signalling molecule cGMP can potently inhibit platelet aggregation and is involved in complex intraplatelet signalling. Regulation of cGMP is mediated through platelet PDE, whilst NO dependent increases in cGMP are mediated through sGC.

To investigate the effect of Sin-1 upon cGMP, the signalling molecule was directly quantified. Intraplatelet cGMP is present in low concentrations, approximately 2000 fmol / 10^9 platelets (Eigenthaler *et al* 1992, Wanstall *et al* 2005). Therefore to observe the effect of Sin-1 on intraplatelet cGMP, platelets were first treated with 100 µM of the PDE inhibitor IBMX. This pretreatment will allow the effect of the stimulation of cGMP without any degradation by present PDE.

cGMP concentrations were quantified in both resting and ADP activated platelets using the Biotrak cGMP EIA (GE Healthcare). After aggregation was induced by ADP, the transient peak of cGMP occurs approximately 10-20 seconds after initial point of activation (Mo *et al* 2004). After 15 seconds, cGMP is reported to be at a peak concentration (Mo *et al* 2004), therefore activation was stopped at this time point and the concentrations of cyclic nucleotides were quantified. The effect of the vehicle control DMSO upon platelet cGMP was also investigated, as isolated platelets were treated with an appropriate amount of diluted DMSO that would equal that used to solubilise ODQ.

Concentrations of cGMP within resting platelets treated with IBMX were measured to be 156 ± 13 fmol and were used as control data. After treatment with Sin-1 for 1 min to remain consistent with aggregation studies, intraplatelet cGMP as expected significantly increased 254 ± 10 fmol from 156 ± 13 fmol of the control baseline level of cGMP. The vehicle control of diluted DMSO showed small but significant increase in cGMP concentrations to 188 ± 3 fmol from 156 ± 13 fmol of the control. The presence of ODQ displayed the same increase in cGMP 192 ± 10 fmol as the vehicle control value of 188 ± 3 fmol, indicating that the presence of the negligible amount of DMSO may be responsible for this elevation. In the presence of ODQ, Sin-1 fails to increase cGMP, as the measured value of 187 ± 9 fmol is consistent with the ODQ alone data of 192 ± 10 fmol. This demonstrates that Sin-1 at 8 µM directly increases cGMP through sGC and that the sGC

inhibitor ODQ is functioning correctly by preventing any increase in concentration of cGMP (Fig. 3.13).

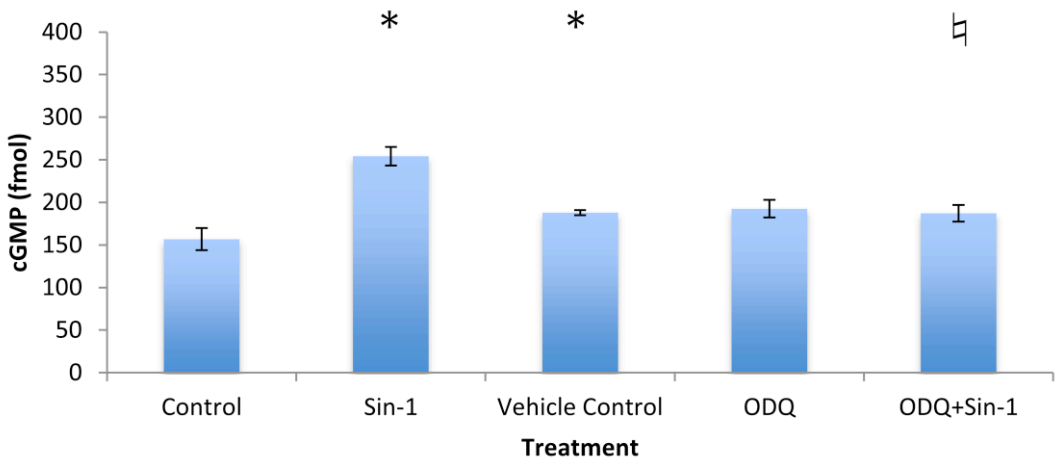


Fig. 3.13:- **cGMP concentrations in the presence of IBMX in resting platelets.** Average intraplatelet cGMP concentrations ± SEM. Platelets were first treated with IBMX. Isolated platelets were further incubated with ODQ before further treatment with Sin-1. Non-ODQ treated resting platelets are represented by the control. Vehicle control shows platelets treated with diluted DMSO, in which the inhibitor ODQ is solubilised. Statistically significant data compared to control are indicated with * above respective point p = 0.041. Statistically significant data compared to Sin-1 data are indicated with ϟ above respective point. All significant data p = 0.037 0.05 and was calculated via students t test (n=4).

ADP activated platelets in the presence of IBMX showed an increase in control cGMP to 263 ± 16 fmol in comparison to cGMP concentrations of 156 ± 13 fmol in platelets in a resting state. Sin-1 significantly increased cGMP to 325 ± 13 fmol from 263 ± 16 fmol in activated platelets, however this increase is smaller than the increase observed in resting platelets. The DMSO vehicle control showed no effect on cGMP concentrations when compared to the control data, whereas ODQ significantly decreased platelet cGMP to 141 ± 10 fmol from 269 ± 8 fmol of the vehicle control. The presence of ODQ abolished the increase in cGMP that Sin-1 can induce significantly decreasing concentrations of cGMP to 114 ± 18 fmol from 263 ± 16 fmol of Sin-1 alone. This indicates that ODQ can effectively prevent an increase in cGMP induced by this low concentration of Sin-1 (Fig 3.14).

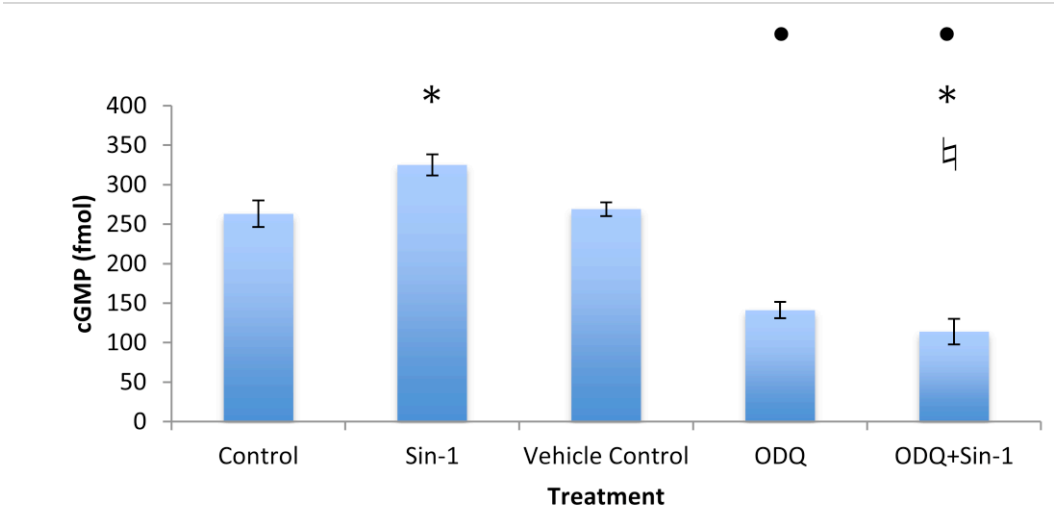


Fig. 3.14:- Average intraplatelet cGMP concentrations in the presence of IBMX and ODQ in ADP activated platelets. Platelet cGMP concentrations ± SEM after platelets were first treated with IBMX, and then ODQ. Platelets were further treated with Sin-1 before aggregation was initiated with ADP. The vehicle control exhibits the effect of diluted DMSO in which ODQ was dissolved. Statistically significant data indicated with * above respective point is significant to control data for Sin-1 has $p = 0.03$ and ODQ + Sin-1 $p = 0.014$. • indicates significance to the vehicle control with $p = 0.023$ and ♮ to Sin-1 data with $p = 0.019$. All data $p \leq 0.05$ and was calculated using students t test (n=4).

As the presence of IBMX may bias the experiments, the investigation was repeated quantifying cGMP without any IBMX present in ADP activated platelets. cGMP in platelets without IBMX showed a diminished basal concentration of 96 ± 20 fmol. Sin-1 significantly increased intraplatelet concentrations to 146 ± 16 fmol from 96 ± 20 fmol. The vehicle control of diluted DMSO had no effect upon cGMP levels as 114 ± 18 fmol is within range of the control data of 96 ± 20 fmol. The presence of ODQ however, completely diminished cGMP concentrations to 4 ± 0.5 fmol from 114 ± 18 fmol of the vehicle data. In the presence of ODQ and Sin-1 platelet cGMP is significantly decreased to an almost imperceptible concentration of 2 ± 1.4 fmol (Fig. 3.15).

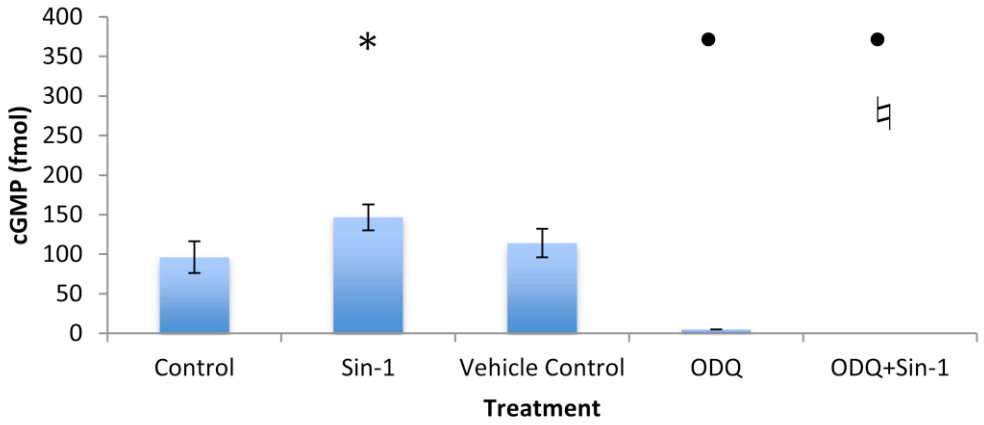


Fig 3.15:- The effect of ODQ upon activated platelets without IBMX. Average intraplatelet cGMP concentrations ± SEM. Platelets were treated with ODQ, before further incubation with Sin-1 before aggregation was initiated with ADP. Statistically significant data indicated with * above respective point is significant to control data p = 0.041. • indicates significance to the vehicle control and ♮ to Sin-1 data p = 0.01. All data was calculated using students t test (n=4).

3.4.2.2 *The effect of PGE_1 upon cGMP*

There is evidence to suggest that there is crosstalk between cGMP and cAMP signalling pathways within platelets including preliminary data presented throughout this thesis. The exact origin of this is currently unknown, with speculation that platelet PDE are responsible (Smolenski 2011). Others suggest that the effect of one cyclic nucleotide on the other is mediated by protein kinase activity (Francis *et al* 2010).

To investigate whether cAMP stimulators can influence cGMP, the effect of the cAMP stimulator PGE_1 was investigated when quantifying platelet cGMP. Isolated washed platelets were incubated firstly with IBMX before being further treated with Sin-1, PGE_1 or both Sin-1 and PGE_1 (final concentrations). Intraplatelet cGMP was then measured using the readily available Biotrak cGMP EIA (GE Healthcare) (Fig. 3.16).

Control platelets indicate intraplatelet cGMP concentrations were 156 ± 13 fmol within resting platelets and that Sin-1 can increase platelet cGMP to 254 ± 10 fmol via the activation of sGC. PGE_1 induced a slight increase in cGMP to 191 ± 15 fmol, however this

increase is not significant. When combined Sin-1 and PGE_1 elevate cGMP to 231 ± 15 fmol by an amount that can be attributed to the effects of Sin-1 alone (Fig. 3.16).

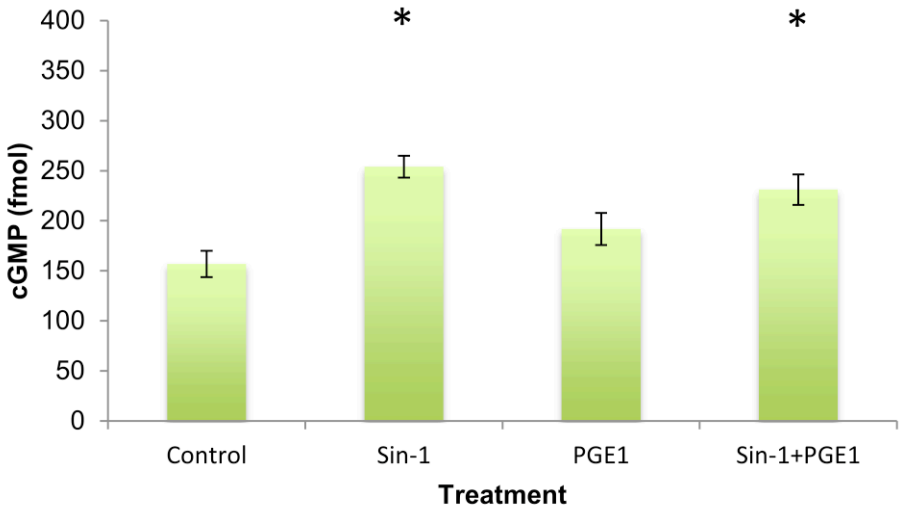


Fig. 3.16:- The effect of cAMP stimulator PGE_1 upon intraplatelet cGMP. Average concentrations of resting platelet cGMP ± SEM after treatment first with IBMX followed by Sin-1 or PGE_1. Statistically significant data compared to the control via students t test are indicated with * above respective point to the control data with p = 0.03 (n=6).

As PDE were inhibited due to the presence of IBMX, this does not examine any cross effect of cAMP upon cGMP due to PDE. These results suggest that cAMP induced activation of protein kinases does not influence cGMP. To investigate this further, it is necessary to quantify cAMP.

3.4.3 Platelet cAMP

3.4.3.1 *The inhibition of AC and the effect upon platelet cAMP*

It is well established that increases in platelet cAMP potently inhibit the activation and therefore the aggregation of platelets (Feijge *et al* 2004). cAMP like cGMP is a secondary messenger used for intraplatelet signal transduction. The membrane bound enzyme adenylyl cyclase catalyses the conversion of ATP into cAMP, whereas the conversion of cAMP into the inactive metabolite AMP is mediated by the cAMP specific PDE3 and PDE2. The presence of the AC inhibitor SQ 22536 removed the inhibitory effects of PGE_1 on platelet aggregation. To investigate the response of cAMP to PGE_1 and SQ 22536, cAMP concentrations were quantified after treatment with both PGE_1 and SQ 22536.

To remove the transient response of the signalling molecule intraplatelet cAMP was quantified in the presence of the PDE inhibitor IBMX thus allowing the full stimulatory response to be observed. To observe the normal response in platelets cAMP was also quantified in the absence of IBMX.

Prostaglandin E_1 potently enhances platelet cAMP and inhibiting AC should remove the elevation in cAMP that PGE_1 generates. Isolated washed platelets were incubated with IBMX and platelets were further incubated with either PGE_1, of the AC inhibitor SQ 22536 or both PGE_1 and SQ 22536. Intraplatelet cAMP was quantified in resting platelet to observe basal concentrations (Fig. 3.17).

Platelet concentrations of cAMP were significantly increased after treatment with PGE_1 to 3091 ± 62 fmol from 1441 ± 195 fmol of the control concentration. cAMP concentrations in the presence of the AC inhibitor SQ 22536 were 1685 ± 315 fmol and similar to the control value of 1441 ± 195 fmol. The presence of SQ 22536 prevented any increase in cAMP provoked by PGE_1, reducing cAMP concentrations to 1576 ± 137 fmol from 3091 ± 62 fmol. This indicates that the increase in cAMP by PGE_1 is mediated through AC and successfully reversed using SQ22536 (Fig. 3.17).

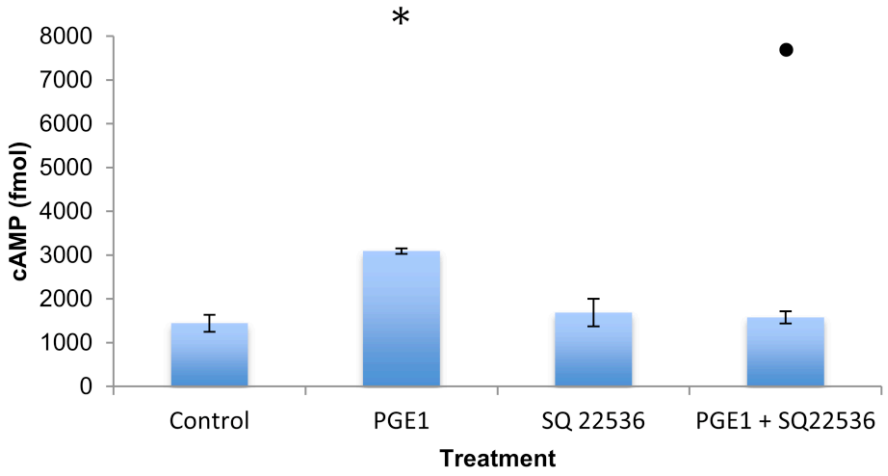


Fig. 3.17:- The effect of PGE_1 upon intraplatelet cAMP in resting platelets. Average concentrations of resting platelet cAMP ± SEM after treatment first with IBMX followed by PGE_1, SQ22536 or both PGE_1 and SQ22536. Statistically significant data compared between treatments via students t test are indicated with * above respective point to the control data with p = 0.032. • indicates significance to PGE_1 data with p = 0.05 (n=4).

The activation of platelets by ADP involves the inhibition of AC by the inhibitory pertussis toxin-sensitive G-protein ($G\alpha_i$), thus causing a decrease in cAMP allowing for aggregation to occur. Control concentrations of cAMP in ADP activated platelets should therefore decrease in comparison to basal levels of cAMP in resting platelets.

Intraplatelet cAMP was determined in ADP activated platelets. Washed platelets were incubated in the presence of IBMX to prevent degradation through active platelet PDE. Platelets were further treated with either PGE_1, the AC inhibitor SQ 22536 or both PGE_1 and SQ 22536. Activation was initiated via the addition of ADP and halted at 15 seconds, a time point at which cAMP concentrations should be optimal (Mo *et al* 2004), by placing the reaction onto ice (Fig. 3.18).

In the presence of IBMX activated intraplatelet concentrations were decreased to 1205 ± 102 fmol when compared to resting platelets of 1441 ± 195 fmol as expected. PGE_1 significantly increased cAMP to 3452 ± 493 fmol from 1205 ± 102 fmol of the control. Interestingly the AC inhibitor SQ 22536 significantly decreased cAMP concentrations to 360 ± 51 fmol from the control concentrations of 1205 ± 102 fmol. This is unusual, as due to the presence of IBMX no PDE should be active and there should remain an endogenous

concentration of cAMP. The presence of SQ 22536 completely abolished the stimulatory effects of PGE_1 upon cAMP reducing concentrations to 421 ± 68 fmol from 3452 ± 493 fmol indicating that SQ 22536 is functioning correctly (Fig. 3.18).

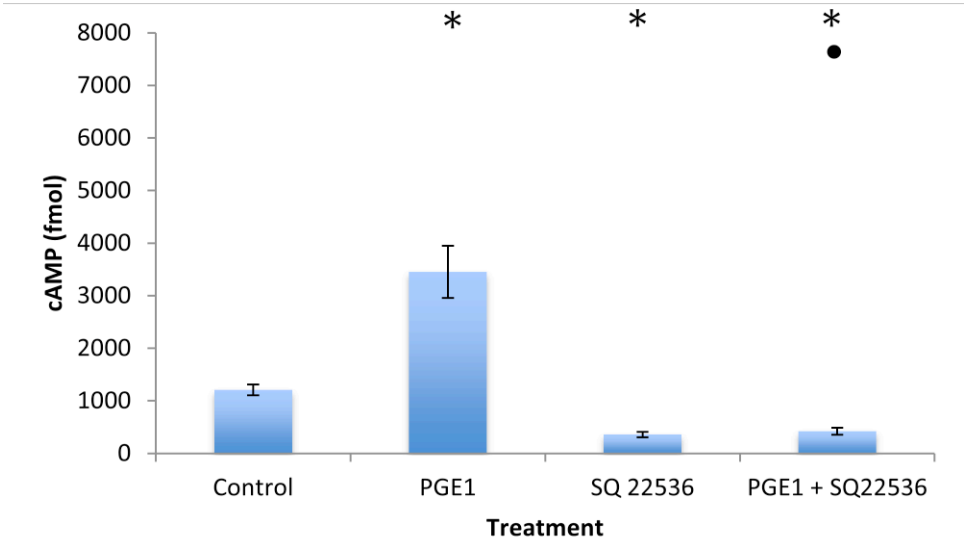


Fig. 3.18:- The effect of PGE_1 upon intraplatelet cAMP in ADP activated platelets with IBMX. Average concentrations of ADP activated platelet cAMP ± SEM after treatment first with IBMX. Platelets were then treated with SQ22536 followed by PGE_1 and the addition of ADP induced activation. Statistically significant data compared between treatments via paired students t test are indicated with * above respective point to the control with p values of 0.02, 0.028, 0.024 for PGE_1, SQ 22536 and PGE_1+ SQ22536 respectively. • indicates significance to PGE_1 data with p 0.02 (n=4).

Intraplatelet concentrations of cAMP were also quantified without IBMX pretreatment to observe the effect of PGE_1 with platelet PDE active. In the absence of IBMX activated platelets exhibited the same response as observed with IBMX, however the concentrations of cAMP are substantially less (Fig. 3.19). PGE_1 significantly increased cAMP to 2784 ± 112 fmol from the control concentration of 860 ± 209 fmol. This significant decrease in cAMP can be explained due to the active platelet PDE degrading cAMP upon ADP stimulation. SQ 22536 noticeably decreased cAMP to 281 ± 36 fmol from the control 860 ± 209 fmol. The presence of the inhibitor continued to remove the

stimulatory effects of PGE_1 decreasing cAMP to 320 ± 46 fmol from 860 ± 209 fmol of PGE_1 alone (Fig. 3.19).

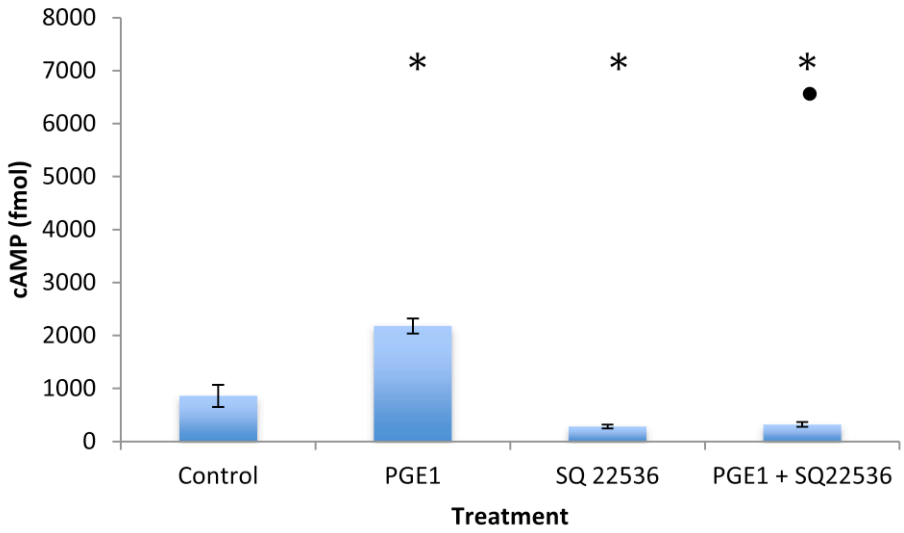


Fig. 3.19- The effect PGE_1 upon intraplatelet cAMP in ADP activated platelets without IBMX. Average concentrations of ADP activated platelet cAMP ± SEM. Platelets were treated with SQ22536 followed by PGE_1 and ADP initiated activation. Statistically significant data compared between treatments via students t test are indicated with * above respective point to the control, with p values of 0.02, • indicates significance to PGE_1 data with p = 0.02 (n=4).

These results highlight the effects of PDE degradation upon platelet cAMP, as without IBMX both cAMP and cGMP display diminished intraplatelet concentrations of each cyclic nucleotide.

There is evidence to suggest that there is a cross effect of one cyclic nucleotide on the other. To investigate this the effect of PGE_1 upon cGMP was investigated, however there was little effect. To thoroughly investigate this the effect of Sin-1 was investigated, to provide support to previous findings by Fisch *et al* 1995.

3.4.3.2 *The effect of Sin-1 upon intraplatelet cAMP*

Intraplatelet cAMP was determined using a readily available Biotrak direct cAMP EIA kit (GE Healthcare). In resting platelets basal cAMP was measured at 1441 ± 195 fmol and was increased significantly by PGE_1 to 3091 ± 62 fmol. The presence of IBMX prevented the degradation of the cyclic nucleotide by platelet PDE. Sin-1, a cGMP stimulator also significantly increased cAMP to 2080 ± 149 fmol from the basal concentration of 1441 ± 195 fmol. Treatment with both PGE_1 and Sin-1 substantially increased intraplatelet cAMP to 7107 ± 439 fmol exceeding the increase induced by cAMP stimulator PGE_1 alone (Fig. 3.20).

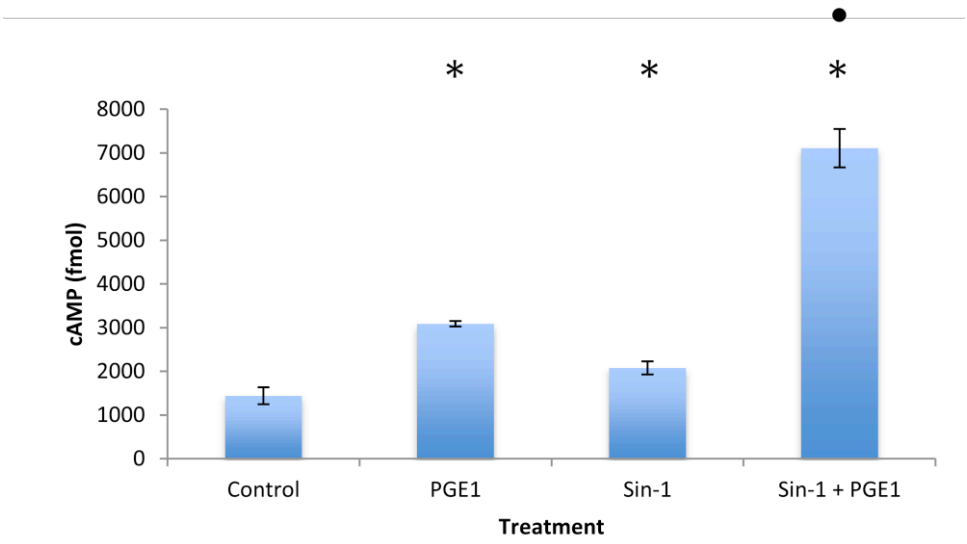


Fig. 3.20:- The effect of Sin-1 upon intraplatelet cAMP in the presence of IBMX. Average concentrations of cAMP in resting platelets ± SEM after treatment with IBMX. Platelets were further treated with PGE_1 or Sin-1. Statistically significant data compared to control between treatments via paired students t test are indicated by * above each respective point to the control with PGE_1 p = 0.02, Sin-1 = 0.03, Sin-1+PGE_1 p = 0.006. • indicates significance to PGE_1 data with p = 0.01 (n=4).

This data suggests that there is synergistic action of PGE_1 and Sin-1 upon cAMP (Fig. 3.20). This data however was observed in the presence of IBMX, therefore any PDE activity is inhibited. It is possible that there is another mechanism further down the

signalling cascade than PDE that may be able to influence cAMP through increases in cGMP.

The effect of Sin-1 was also investigated in ADP activated platelets, both in the presence (Fig. 3.21) and absence of IBMX (Fig. 3.22). Isolated platelets were first incubated with or without IBMX before being challeneged with PGE_1, Sin-1 or both PGE_1 and Sin-1. Activation was initiated via the addition of ADP and halted at approximately 15 seconds by placing the reaction onto ice. Intraplatelet cAMP was determined using a Biotrak direct cAMP EIA kit (GE Healthcare).

In the presence of IBMX (Fig. 3.21), control concentrations of cAMP in ADP activated platelets were measured as 1205 ± 102 fmol and were significantly increased by PGE_1 to 3452 ± 493 fmol. Sin-1 failed to induce a response in ADP activated platelets with cAMP concentrations of 1174.6 ± 75 fmol similar to the control concentrations of 1441 ± 195 fmol. Interestingly the same synergistic effect of PGE_1 and Sin-1 upon intraplatelet cAMP as observed in resting platelets was also present in activated platelets. The combination of the AC and sGC activators significantly increased intraplatelet cAMP to 4175 ± 48 fmol again excedding what PGE_1 alone can induce (Fig. 3.21).

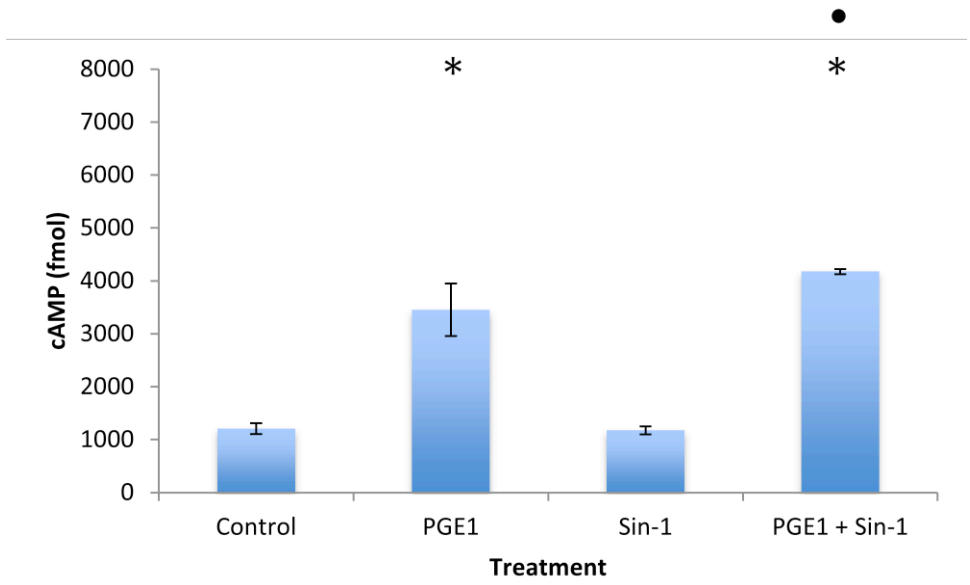


Fig. 3.21- The effect of Sin-1 upon intraplatelet cAMP in ADP activated platelets with IBMX. Average concentrations of cAMP in ADP activated platelets ± SEM in the presence of IBMX treatment. Platelets were then treated with either PGE_1 or Sin-1 with ADP inducing platelet activation. Statistically significant data compared between treatments via paired students t test are indicated with * above respective point to the control with a p value of p = 0.03. • indicates significance to PGE_1 data with p = 0.048 (n=4).

Platelet cAMP in activation platelets without IBMX was also established (Fig. 3.22), with basal concentrations of cAMP of 860 ± 209 fmol decreased due to the uninhibited activity of platelet PDE. PGE_1 significantly increased control concentrations to 2784 ± 112 fmol from 860 ± 209 fmol. Platelets treated with Sin-1 also significantly raised concentrations of cAMP to 1634 ± 309 fmol from 860 ± 209 fmol of the control.

The presence of both PGE_1 and Sin-1 considerably increase intraplatelet cAMP concentrations to 3343 ± 189 fmol exceeding the response observed from PGE_1 alone (Fig. 3.22).

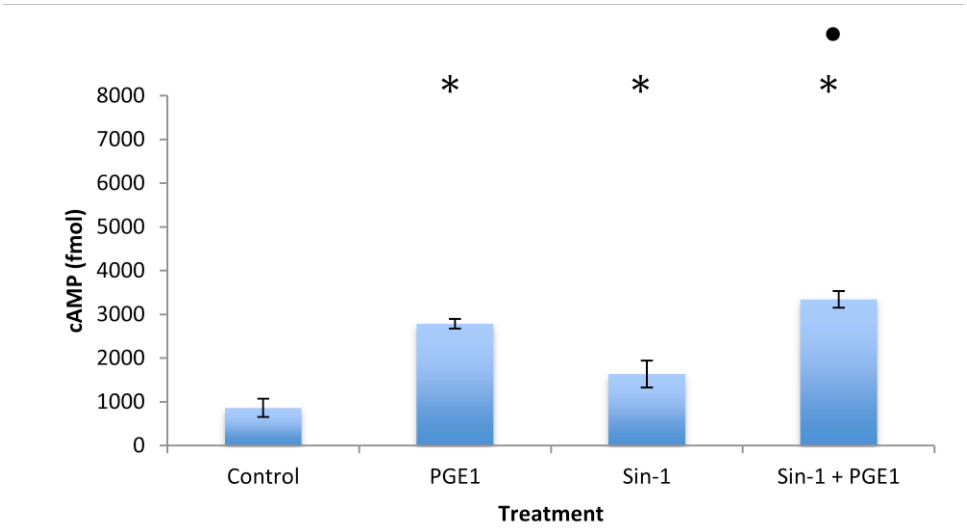


Fig. 3.22:- The effect of Sin-1 upon intraplatelet cAMP in ADP activated platelets in the absence of IBMX. Average concentrations of ADP activated platelet cAMP ± SEM. Platelets were treated with either PGE_1 or Sin-1 and ADP induced platelet activation. Statistically significant data compared between treatments via paired students t test are indicated with * above respective point to the control with p = 0.036. • indicates significance to PGE_1 data with p = 0.05 (n=4).

The absence of IBMX in this data (Fig. 3.22) highlights the synergistic action of PGE_1 and Sin-1 upon intraplatelet cAMP. This corroborates observations that Fisch detailed; Sin-1 can increase cAMP in the presence of an AC activator, such as PGE_1. With no IBMX present, it is likely that this synergistic increase is due to the action of cGMP causing a negative effect on platelet PDE3A allowing an increase in cAMP.

3.4.4 Disaggregation of ADP induced platelet aggregates via increasing cyclic nucleotides

Increases in cAMP and cGMP are potent inhibitors of platelet aggregation, with the addition of stimulators of either cyclic nucleotide also able to disperse agonist induced platelet aggregates into single platelets. Reagents that can increase platelet cyclic nucleotides or reduce the expression of the GPIIb/IIIa receptor have displayed an ability to induce the disaggregation of platelets (Huang and Hellems 1993, May *et al* 1998)

To investigate the disaggregation effects of cGMP and cAMP, platelets were treated with Sin-1 and PGE_1 respectively with ADP initiating platelet aggregation. The reaction ran until aggregation ≥ 65% was achieved, before Sin-1 and PGE_1 were added to the aggregated PRP. The reaction was allowed to run for 8 min, before data was collected and analysed, at which point sufficient disaggregation was achieved. Longer time spans were also investigated, however the majority of reactions had reached maximal disaggregation after 8 mins (Fig. 3.23a).

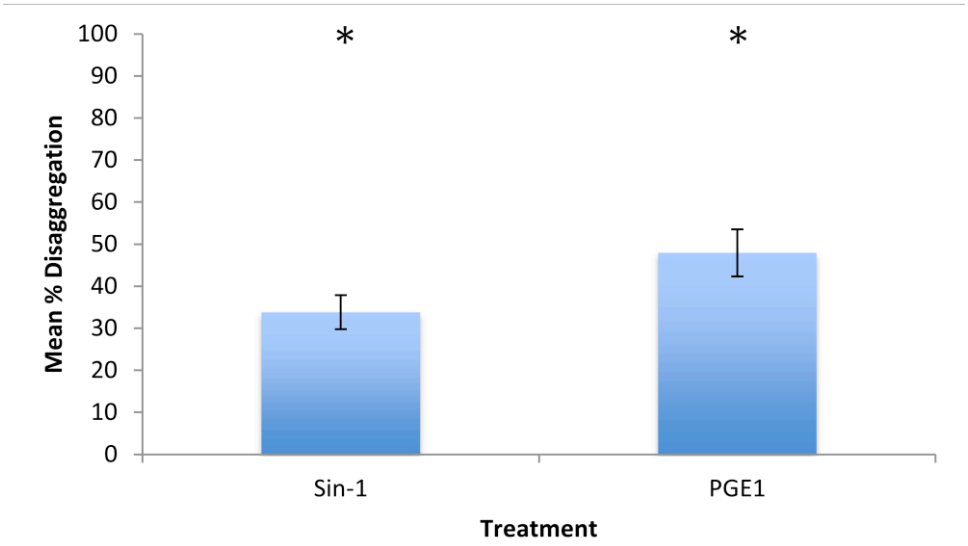


Fig. 3.23a:- The disggregatory effects of Sin-1 and PGE_1 on ADP induced platelet aggregation *in vitro*. Aggregation was initiated in PRP via the addition of ADP. Once platelets had achieved ≥ 65% aggregation, Sin-1 or PGE_1 was added to PRP and left for a total of 8 min. Disaggregation values presented are calculated to the control data. Statistical analysis was calculated between Sin-1 and PGE_1 using a students paired t test with $p = 0.045$ (n=6).

The addition of Sin-1 to the pre-aggregated PRP caused disaggregation of 33 % ± 4 %. PGE_1 when added to the aggregated PRP caused significantly more disaggregation than Sin-1 increasing the disaggregation of platelets to 47 % ± 5 %. This data suggests that an increase in cAMP has more of an inhibitory effect on platelets than increases in intraplatelet cGMP. This can also be observed from the aggregation curves, as the rate of disaggregation after the addition of Sin-1 is much slower when compared to PGE_1 treated PRP (Fig. 3.23b).

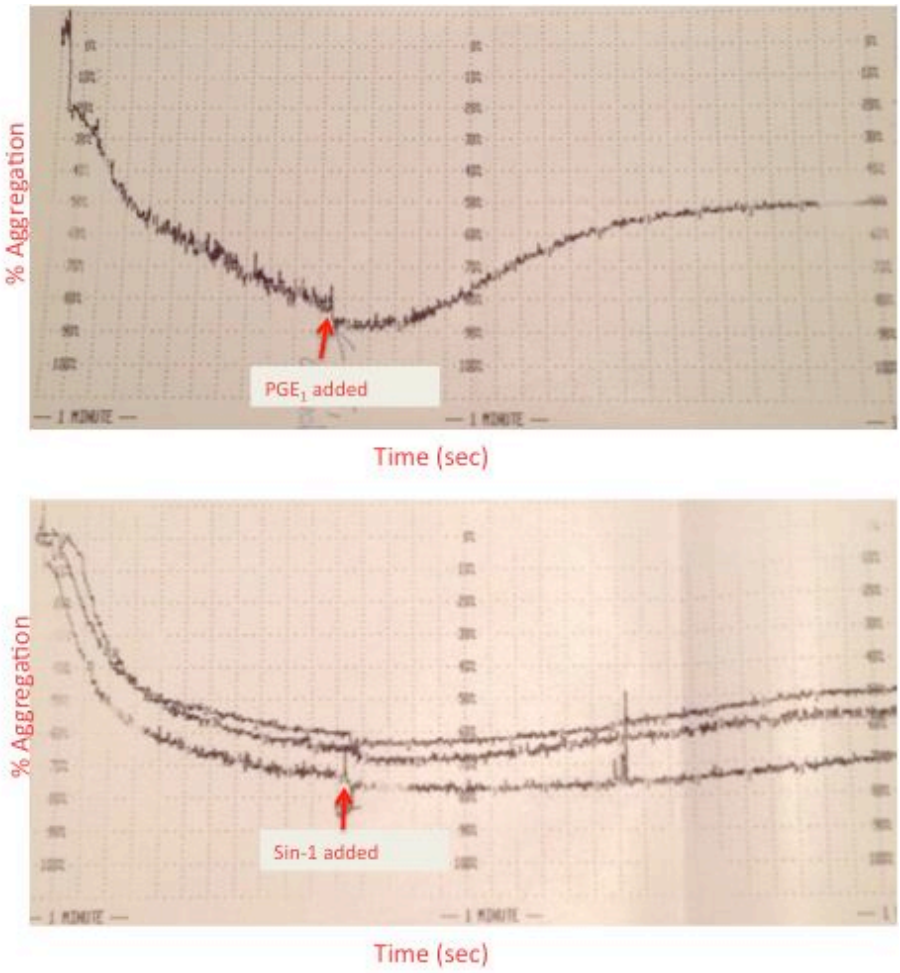


Fig. 3.23b:- Comparison of the disaggregation of platelets caused by Sin-1 and PGE_1 on ADP induced platelet aggregation *in vitro*. Aggregation was initiated in PRP via the addition of ADP. Once platelets had achieved ≥ 65% aggregation, Sin-1 or PGE_1 was added to PRP and left for a total of 8 min. The addition of Sin-1 and PGE_1 are indicated (n=6).

When comparing the curves of disaggregation, the rate of disaggregation indicates that increases in cAMP has a much more potent inhibitory effect upon platelets than cGMP. This also could be attributed to the ability of cGMP to influence cAMP concentrations through either platelet PDE or PK.

As cGMP is an important mediator of platelet function, and NO induces synthesis of the cyclic nucleotide, the effect of NO upon platelets was investigated.

3.4.5 Nitration of platelet proteins

3.4.5.1 *Measurement of nitrite using the Griess assay*

The chemistry of NO is complex with many metabolites of NO found *in vivo* such as nitrite and nitrate. Nitrite itself is an effective vasodilator, however the majority of the inhibitory effects of nitrite upon platelets appears to be mediated through its reduction to NO (Lundberg *et al* 2005, Srihirun *et al* 2012).

The measurement of NO directly is difficult and requires specialist equipment and techniques. Monitoring nitrite can provide an indication to RNS metabolism and the activity of NO *in vivo*. For the purpose of this investigation the detection of nitrite using a simple assay, can provide an insight into the activity and production of platelet NO from isolated platelets. The griess assay is a simple but crude *ex vivo* measurement of nitrite with a detection limit of 3μM. To establish the concentrations of NO from platelet extracts, platelets nitrite was measured before and after treatment with Sin-1.

Isolated platelets were treated with PBS for the negative control and Sin-1 as positive control as treatment with Sin-1 should invoke an increase in nitrite concentration. Treated platelets were lysed and the resulting platelet lysate and media were kept separate and each analysed individually for nitrite concentrations using the griess assay.

Nitrite concentrations within the lysate sample were very low with both the control of 0.9 ± 0.1 μM and the Sin-1 treated sample at 3.4 ± 0.5 μM of concentrations that are beyond the 3 μM detection sensitivity limit of the assay (Fig. 3.24). The data from the washed platelet lysate therefore cannot be relied upon as accurate therefore no statistical analysis was performed.

Within the media samples, nitrite concentration was significantly increased after treatment with Sin-1 to 20.6 ± 3.5 μM from the control of 5 ± 0.9 μM. The results from the media samples are still lower than expected, especially after treatment with Sin-1, suggesting that the data generated from these experiments is not entirely accurate (Fig. 3.24).

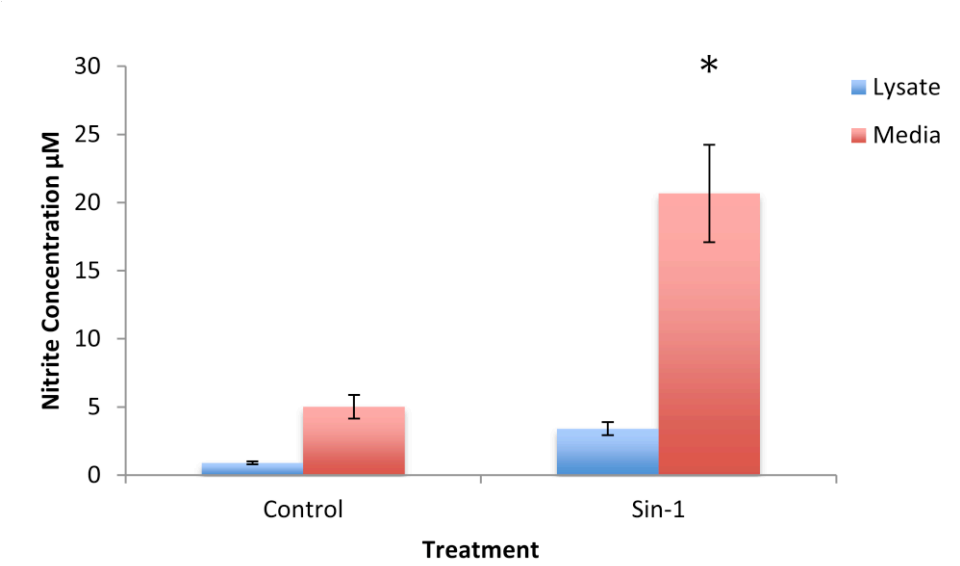


Fig. 3.24:- Measurement of nitrite in platelet lysates. Total nitrite concentration ± SEM in platelet lysates (blue) and media (red) in untreated platelets and positive control Sin-1. Platelets were treated with PBS or Sin-1 before being lysed. Platelet lysate and media were separated and tested for nitrite individually. Statistical significance was not calculated on the lysate samples due to small sample number and the results being below the detection limit of the assay (n=3). Platelet media sample significance is highlighted by * to the control data calculated via students t test p=0.006 (n=6).

To further investigate platelet RNS metabolites, another NO metabolite, platelet nitrotyrosine was quantified.

3.4.5.2 *Platelet Nitrotyrosine*

Modification of proteins through phosphorylation or nitration can have an important role within intracellular signalling cascades. Recent evidence suggests that the nitration of platelet tyrosine residues has a role in intraplatelet signalling. The nitration of the amino acid tyrosine producing nitrotyrosine can serve as another marker for NO activity *in vivo*. Not all proteins are targeted for nitration, neither are all tyrosine residues in target proteins nitrated (Marcondes *et al* 2006). Generation of NO and the presence of $O_2^{\bullet-}$ can combine to produce peroxynitrite, with the superoxide also capable of nitrating proteins (Upmacis 2008).

Human platelets can themselves produce NO and $ONNO^-$ with the presence of nitrotyrosine thought to be vital in the transduction of key signalling (Marcondes *et al* 2006). Platelets treated with Sin-1 would be expected to have an increased expression of nitrotyrosine, due to the generation of both NO and $ONNO^-$ and may therefore act as a positive control. The purpose of these experiments was to identify concentrations of platelet nitrotyrosine to provide an indication of NO activity *ex vivo*.

To establish a correct methodology and whether the chosen antibodies were specific, the protein human serum albumin (HSA) was treated excessively with Sin-1 to induce nitration of the protein. The methodology was verified using the nitrated HSA before using platelet proteins. The primary antibody was tested for specificity by adding excess 3-nitrotyrosine (Sigma Aldrich) during the primary antibody incubation period. No bands were observed on development, indicating that the antibody was binding specifically to nitrotyrosine.

Isolated platelets were treated with PBS (negative control) or Sin-1. Crude lysates were made of the appropriate treated platelets and proteins were separated using SDS PAGE. Separated proteins were then transferred to nitrocellulose and probed using a monoclonal mouse anti nitrotyrosine antibody (Santa Cruz). A separate 15 % gel with the corresponding proteins loaded was stained with coomassie brilliant blue. The expected weight of nitrotyrosine is 67 kDa, as indicated with the nitrated HSA (Fig. 3.25a), whereas the developed bands from the platelet samples displayed a molecular weight of approximately 100 kDa (Fig. 3.25b).

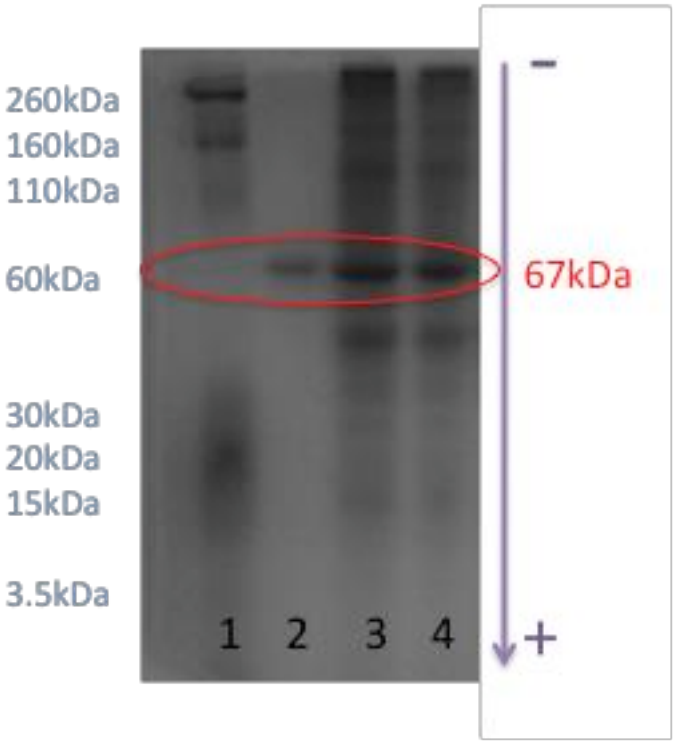


Fig. 3.25a:- Comassie stained SDS PAGE indicating extracted platelet proteins. The highlighted 3 bands indicate 67 kDa size. An appropriately sizes prestained marked (1) was run in lane 1 with the second lane, with a single band, indicating a control nitrotyrosine protein (2). The third lane has separated proteins from untreated platelets (3), with the fourth lane showing proteins from platelets treated with Sin-1 (4).

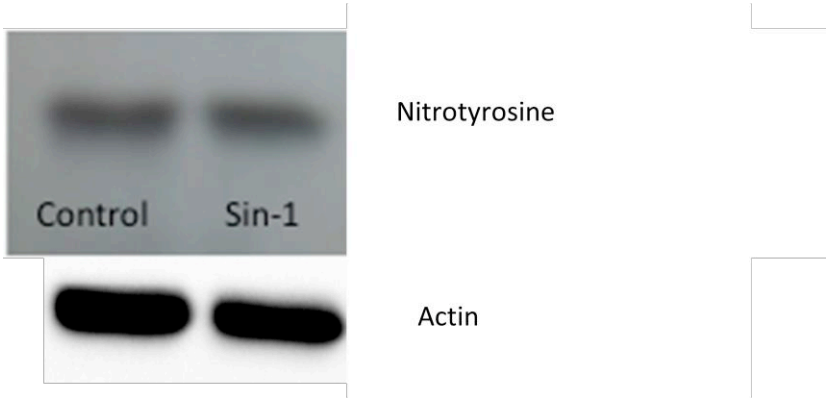


Fig.3.25b:- Western blot indicating nitrotyrosine in lysed platelets. Untreated platelets (P), compared to positive control Sin-1 (PS) (final concentration). All platelets were treated before being lysed and 40µg of protein per lane was loaded onto a 15 % SDS gel. Once separated, proteins were transferred to PVDF membrane and probed with a nitrotyrosine specific antibody. Actin shows the loading control, indicating the same amount of protein was present per sample.

Untreated platelets displayed moderate concentrations of nitrotyrosine whereas platelets treated with Sin-1 unexpectedly did not significantly increase expression of the nitrated protein. An actin loading control was also utilised to confirm the same amount of protein was loaded per lane. The increased molecular weight of the nitrated protein was identified as the nitrated cytoskeleton protein α-actinin isoform 1.

As α-actinin is an important scaffolding protein providing a structure for signalling proteins to transverse as well as being involved in the restructuring of platelets upon activation, platelet shape change was also investigated.

3.4.6 Scanning electron microscopy imaging of ADP induced platelet shape change

Upon stimulation with ADP, platelets undergo a series of shape changes that lead to the formation of an aggregate. After agonist induced activation the initial response is for platelets to change shape reorganising the cytoskeleton culminating in spheration, generation of pseudopodia and ultimately spreading of the platelet (Jagroop *et al* 2000).

To assess how the inhibition of aggregation induced by NO and increases in cAMP and cGMP affects this process, platelets were imaged using scanning electron microscopy (SEMic) over a time course after treatment with Sin-1 and PGE_1 to monitor the shape change before and during aggregation. SEMic is a type of electron microscopy that images a sample by scanning via a high energy of electrons. Imaging requires the samples to be electrically conductive therefore after fixation samples are coated with an ultrathin layer of a conductive material.

Isolated platelets were treated with either PBS (control) or Sin-1 or PGE_1. An aliquot of platelets was taken per treatment to indicate control resting platelets, termed time 0 sec. Platelets were activated by ADP with aliquots taken at 10, 30 and 60 seconds after activation was initiated and fixed. Platelets were imaged at 10,000 X under high vacuum at 12.5 kv (Fig. 3.26 - 3.28).

The control sample (Fig. 3.26), at time 0 displays resting inactive platelets discoid in shape. Platelets 10 seconds after activation by ADP exhibit filopodia as well as an

increased size indicating the flattening of the platelets for spreading. Platelets have spread further 30 seconds after activation, adhering to each other displaying the initial forming of an aggregate. At 60 seconds, a fully formed platelet clot is visible, with single individual platelets impossible to identify (Fig. 3.26).

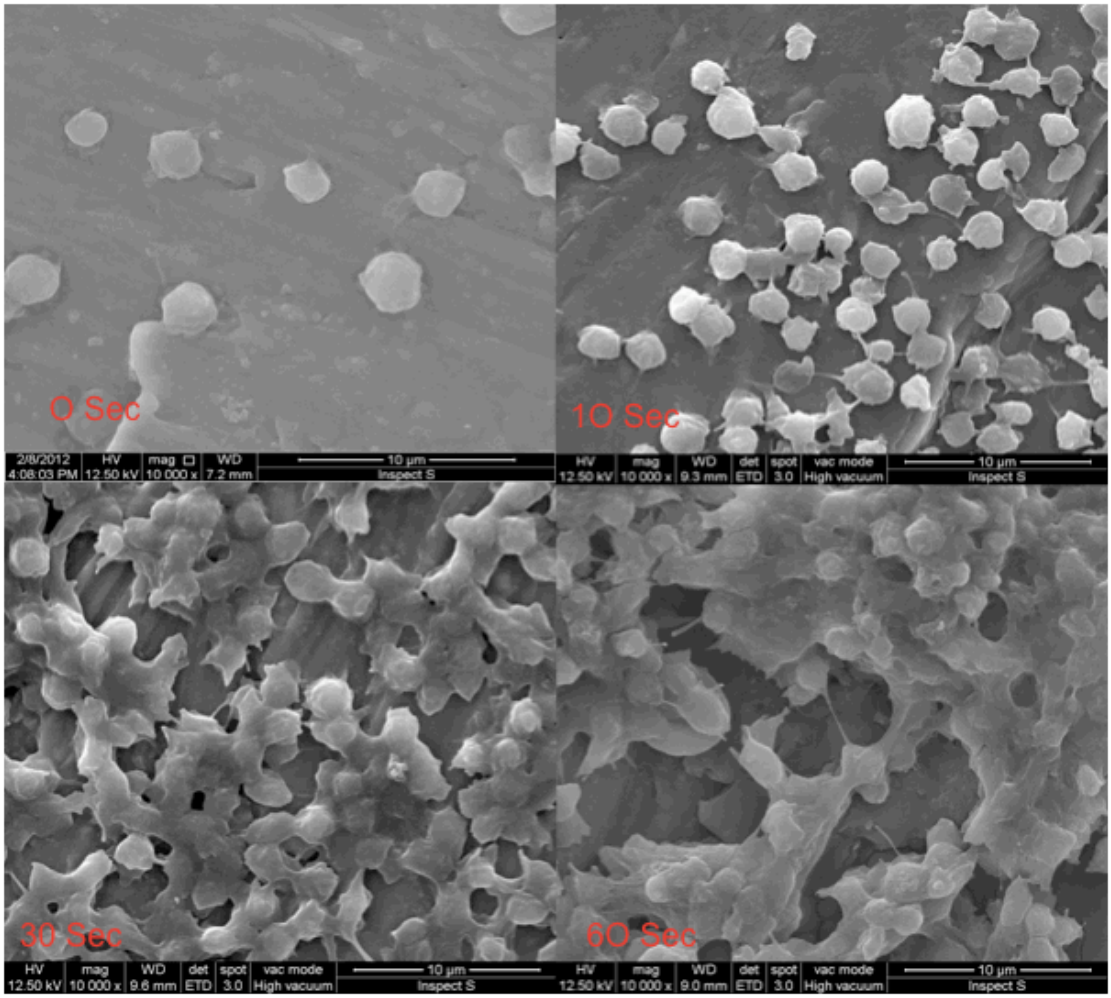


Fig. 3.26:- SEMic image of ADP induced activated platelets. Control, showing platelets before aggregation (0 sec), then at time periods of 10 sec, 30 sec and 60 sec after aggregation was initiated with ADP. Platelets before the addition of ADP can clearly be observed to be in their unstimulated discoid shape. After activation, the platelets begin to flatten and form pseudopodia, eventually forming a fully formed clot and entirely losing their spherical shape by 60 seconds (60 sec).

Platelets treated with Sin-1 show some activation (Fig. 3.27), however there are some platelets that remain in their inactive discoid shape across the time course, despite the addition of the agonist ADP. Treatment of the platelets with Sin-1 will cause an

increase in intraplatelet cGMP, inhibiting platelet aggregation. Aggregation studies displayed approximately 38 % inhibition of aggregation after platelets were treated with Sin-1. At resting time 0 and time 10 seconds after platelet activation by ADP, platelets remain in their inactive spherical shape. Platelets at 30 sec and 60 sec however showed activation with a small percentage of platelets starting to migrate together to form an aggregate. These images corroborate aggregation studies, indicating that there is still some activation and aggregation of platelets that have been treated with a low concentration of Sin-1 (Fig. 3.27).

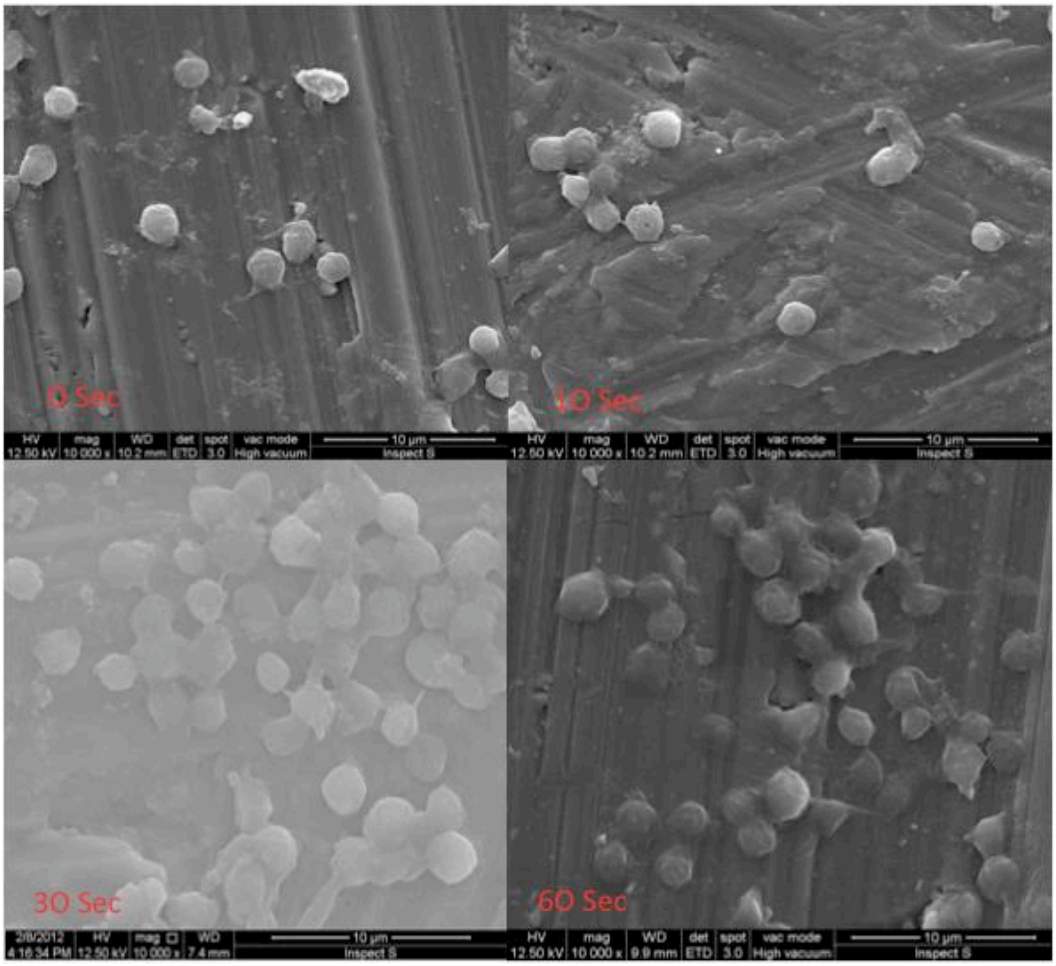


Fig. 3.27:- SEMic images indicating the effect of platelets treated with Sin-1 upon ADP activation. Platelets were first treated with Sin-1 before aggregation was initiated with ADP. Resting platelets before aggregation at 0 sec, then imaged at time periods of 10 sec, 30 sec and 60 sec after aggregation was initiated.

Treatment of platelets with PGE_1 (Fig. 3.28) will provoke an increase in intraplatelet cAMP, therefore inhibiting platelet aggregation. Time 0 indicates the inactive

discoid shape of platelets pre-treated with PGE_1. The addition of ADP fails to induce platelet aggregation as observed with the control sample. Some activation of platelets can be observed at 30 sec and 60 sec, with pseudopods visible from few of the imaged platelets. Despite some activated platelets, a fully formed aggregate was never observed during the course of imaging (Fig. 3.28).

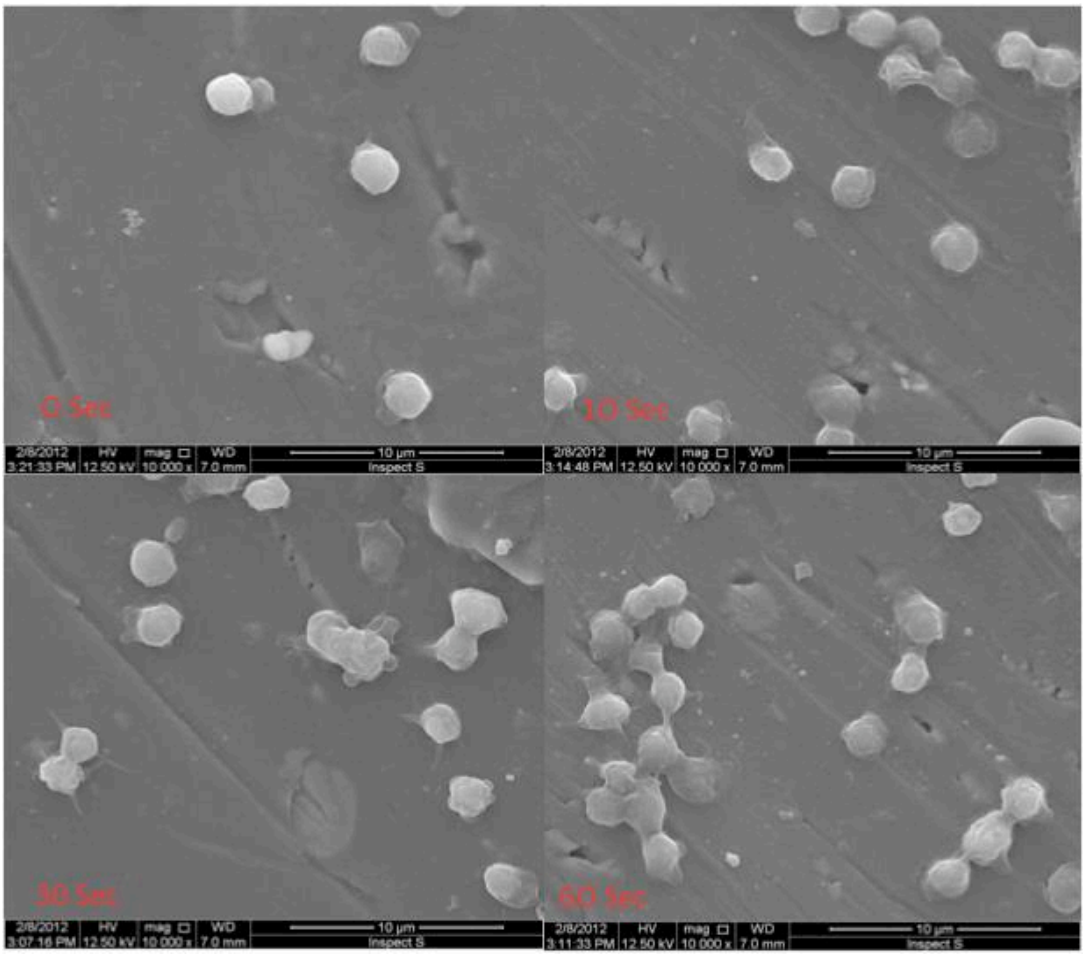


Fig. 3.28:- SEMic images indicating the effect of platelets treated with PGE_1 upon ADP induced activation. Platelets were first treated with PGE_1 before aggregation was initiated with ADP. Resting platelets before aggregation (0 sec), and at time periods of 10 sec, 30 sec and 60 sec after aggregation was initiated.

In aggregation studies, treatment of platelets with PGE_1 caused approximately 67% inhibition of aggregation, provoking more inhibition of aggregation than treatment with Sin-1 at 38 % inhibition. This is also observed during the course of imaging, with more activated platelets visible after treatment with Sin-1 than platelets treated with PGE_1.

These images along with aggregation and disaggregation studies suggest that cAMP has a more potent inhibitory effect upon platelets than cGMP. Increases in either cyclic nucleotide can induce downstream signalling that will inhibit the reorganisation of the actin cytoskeleton; to observe this platelet actin was fluorescently labeled and imaged using fluorescent microscopy.

3.4.7 Platelet actin structure during ADP activation

The formation of filipodia in platelets upon activation requires the reorganization of the platelet cytoskeleton. This remodeling of the platelet from its inactive discoid shape to amorphous, involves the destruction of the actin skeleton. To observe the change in actin structure after ADP stimulation and whether treatment with Sin-1 or PGE_1 had any effect, platelets were fixed, permeabilised and labeled with a fluorescent anti-actin specific antibody. Isolated platelets were treated with PBS (control), Sin-1 or PGE_1. An aliquot of resting platelets, time 0 was removed from each treatment before platelets were activated by the addition of ADP. An aliquot of each treatment was taken at 10, 30 and 60 seconds after activation and fixed. Samples were labeled with a mouse monoclonal anti-actin FITC conjugated antibody and imaged using a fluorescent microscope at 100 x. Each image is a representative sample per treatment and time point (Fig. 3.29 – 3.31).

Control platelets are observed to be in a resting inactive discoid shape at time 0 (Fig. 3.29). After activation with ADP, platelets appear larger indicating flattening, with pseudopodia generated visible from some platelets at 10 sec. At 30 seconds after activation, platelets have more pseudopodia with some adhering to nearby platelets. The leading edges with forming pseudopodia are brighter showing more concentrated levels of platelet actin, with platelets also visibly congregated together yet individual cells still identifiable. At 60 seconds after activation, a fully formed clot is present. These images show the change in actin structure that occurs during platelet shape change (Fig. 3.29).

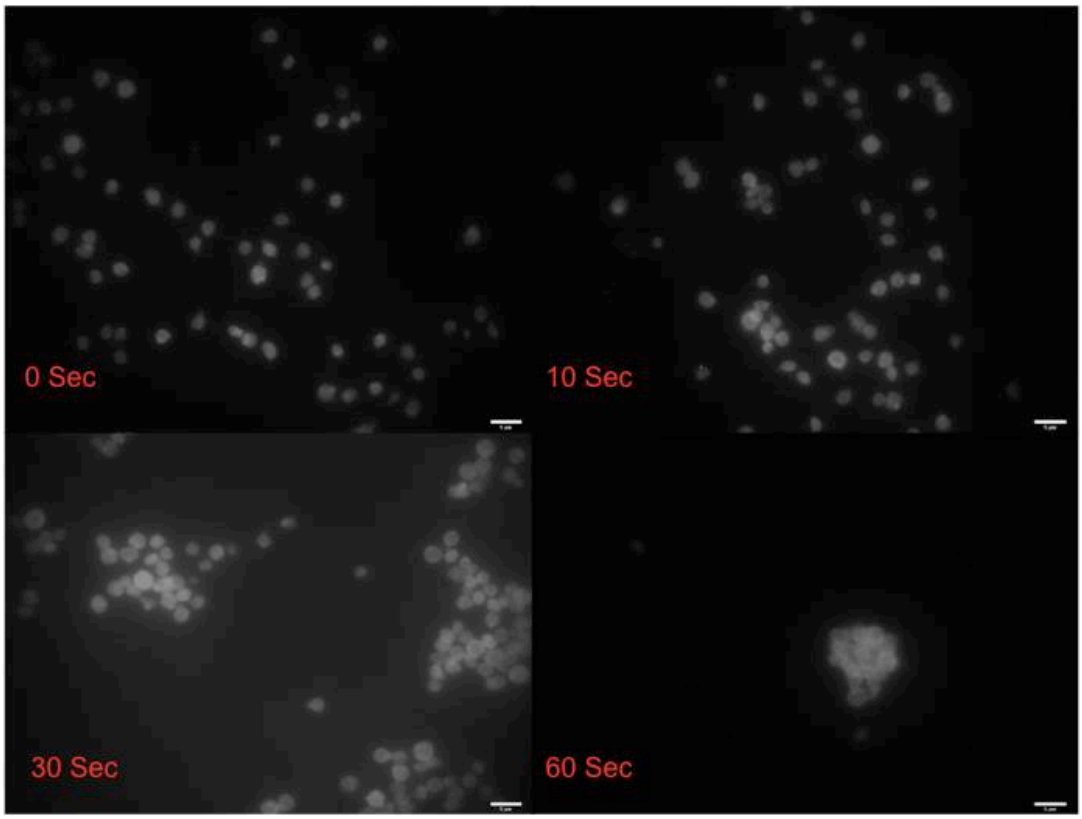


Fig. 3.29:- The effect of ADP induced shape change upon platelet actin. Fluorescently labeled images of platelet actin at 100x. Control showing fluorescently actin labeled platelets before aggregation (0 sec), then at time periods of 10 sec, 30 sec and 60 sec after aggregation was initiated with ADP.

Platelets treated with Sin-1 mostly remain in their inactive shape due to the increased intraplatelet cGMP (Fig. 3.30). There are few cells that have filopodia formed, with these platelets also displaying a leading edge of actin for re-organization. The inactive platelets have a relatively consistent brightness across the cell, indicating the resting state of the platelet, as the actin cytoskeleton remains unchanged. A small aggregate is beginning to form 60 seconds after activation with ADP in Sin-1 treated platelets. As Sin-1 only induces 38 % inhibition of aggregation there are still platelet aggregates formed and this is exhibited by the small amount of platelets migrating towards other platelets 60 seconds after activation (Fig. 3.30).

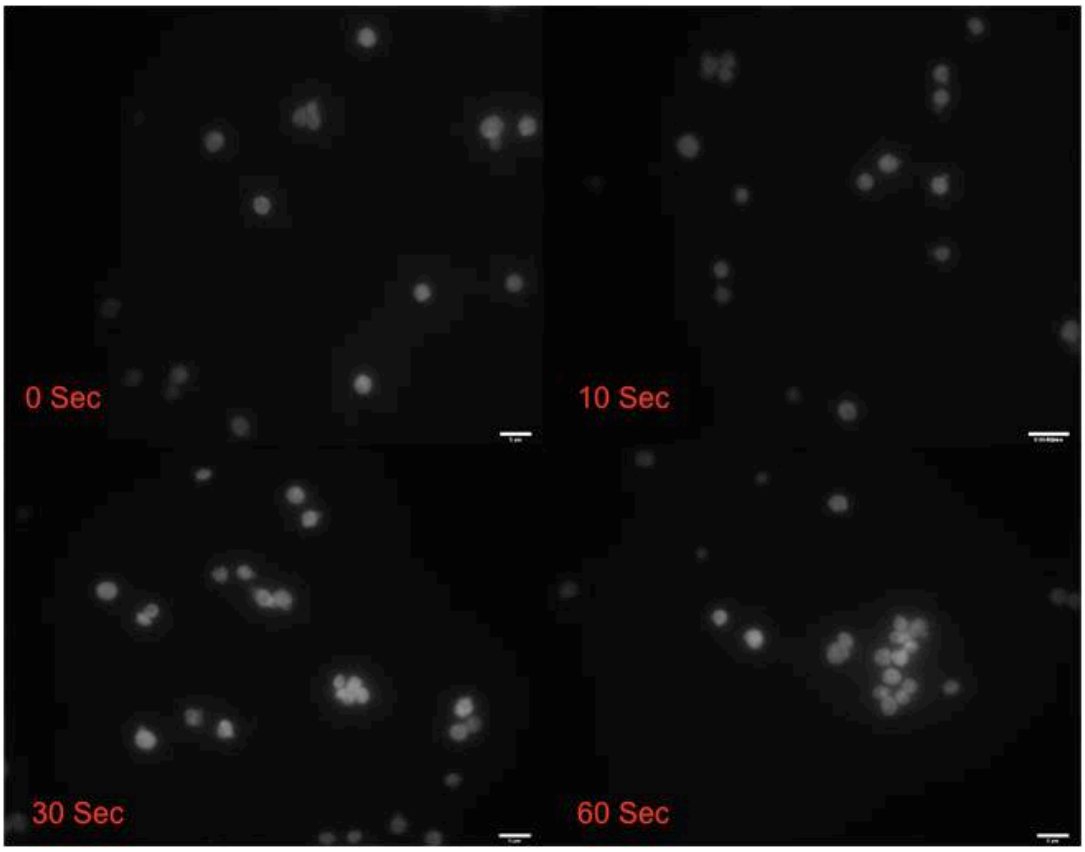


Fig. 3.30:- The effects of Sin-1 upon actin in ADP induced platelet shape change. Fluorescently labeled images of platelets at 100x. Sin-1 control indicates fluorescently actin labeled platelets before aggregation (0 sec), then at time periods of 10 sec, 30 sec and 60 sec after aggregation was initiated with ADP.

PGE_1 treated platelets will have an increased concentration of intraplatelet cAMP. Compared to Sin-1, there are fewer activated platelets visible across the time course despite activation by ADP as the majority of treated platelets remain in their inactive spherical shape (Fig. 3.31).

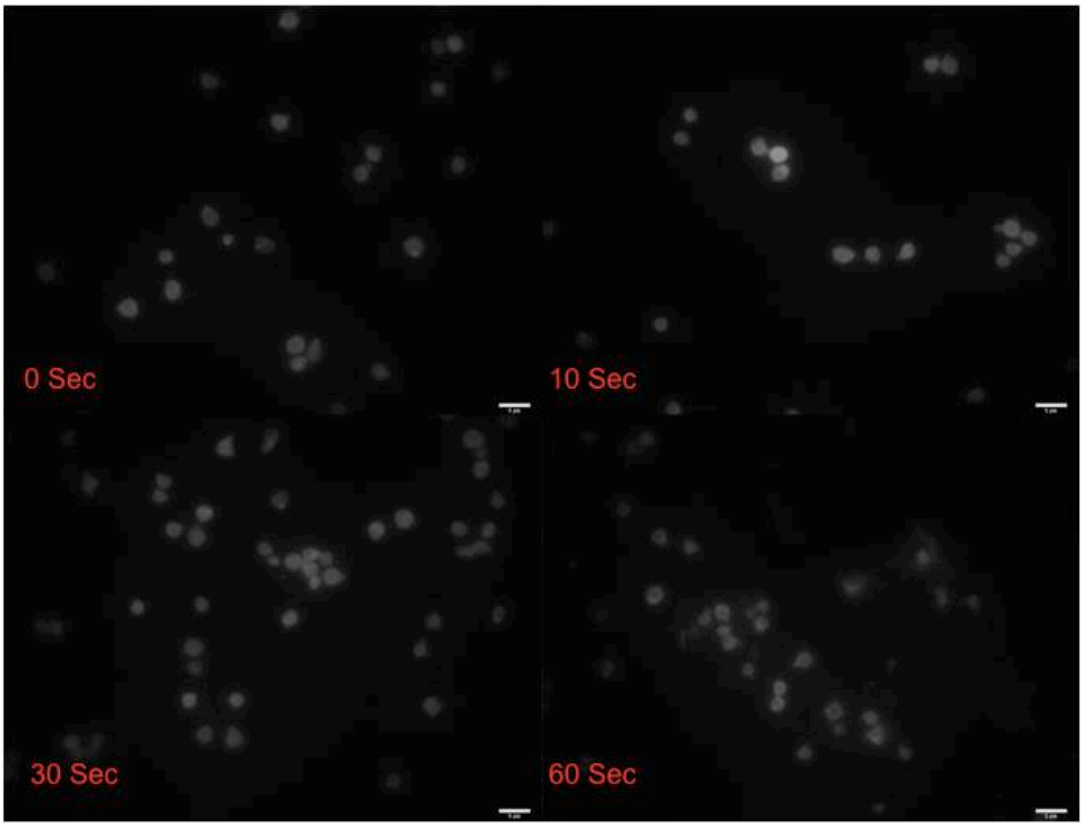


Fig. 3.31:- The effects of PGE_1 upon actin in ADP induced platelet shape change. Fluorescently labeled images of platelets at 100x. PGE_1 treatment control displays fluorescently actin labeled platelets before aggregation (0 sec), then at time periods of 10 sec, 30 sec and 60 sec after aggregation was initiated with ADP.

Increases in intraplatelet cyclic nucleotides inhibit aggregation, shape change and the reorganization of the platelet cytoskeleton. The binding of fibrinogen by the platelet receptor GPIIb/IIIa is a crucial step in stablising a platelet aggregate irrelevant of the initial agonist. Increasing cGMP or cAMP will reduce the expression of GPIIb/IIIa and should therefore decrease the binding of platelets to fibrinogen.

3.4.8 The effect of NO upon platelet adhesion to fibrinogen

The binding of fibrinogen is a common step in all agonist induced platelet activation. Binding is the last step during platelet aggregation and is due to the adherence to exposed fibrinogen by the glycoprotein receptor GPIIb/IIIa, also termed $\alpha_{IIb}\beta_3$ intergrin. Upon activation the expression of GPIIb/IIIa is increased and converted to a high affinity form, therefore binding more fibrinogen to stabilise the aggregate

To assess the effects of NO upon platelet $\alpha_{IIb}\beta_3$ and fibrinogen interactions a simple assay using immobilized fibrinogen was utilized. This assay utilises immobilised fibrinogen and the permeable stain Rose Bengal. Platelets that have adhered to the fibrinogen will be lysed thus releasing the absorbed Rose Bengal solution. The measured absorbance of the Rose Bengal will be proportional to the amount of platelets bound to fibrinogen.

Isolated washed platelets were treated with PBS, Sin-1 or of 4-[4-[4-(Aminoiminomethyl) phenyl]-1-piperazinyl]-1-piperidineacetic acid trihydrochloride (GR144053) a potent inhibitor of the GPIIb/IIIa receptor. Platelets treated with GR144053 will therefore stand as a negative control, with PBS treated the positive control. A 96 well plate was coated with fibrinogen before the respective treated platelets were added. Platelets were either left in a resting state to observe any spontaneous binding of platelets (Fig. 3.32) or activated by ADP (Fig. 3.33).

In resting platelets, there will be a small amount of spontaneous binding, as the low affinity form GPIIb/IIIa will also bind fibrinogen. This binding however is negligible; despite this treatment of resting platelets with Sin-1 still significantly decreases the binding to fibrinogen (Fig. 3.32).

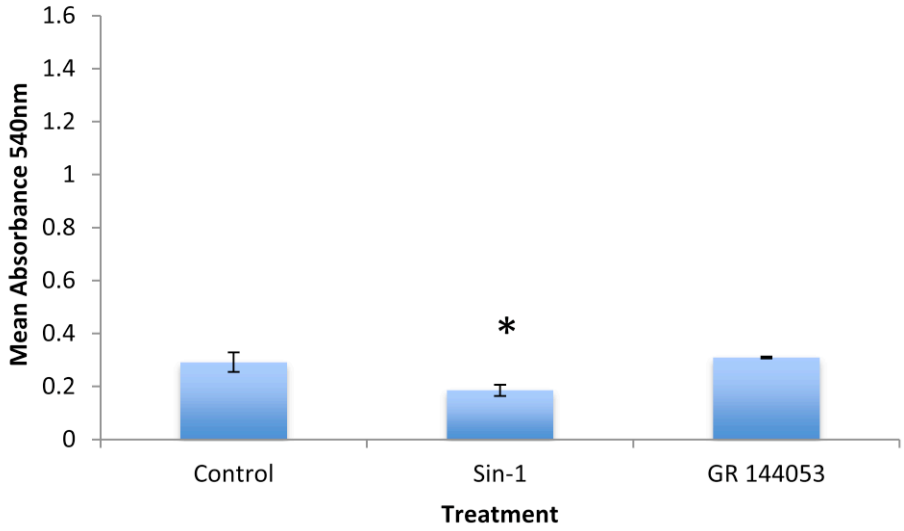


Fig. 3.32:- The effect of NO upon resting platelets binding to immobilised fibrinogen. Platelets were either not treated, treated with either Sin-1, or GR 144053, before being added on the fibrinogen coated plate. Statistical analysis calculated to untreated control platelets via paired students t test and indicated via * over respective point with p = 0.04 (n=6). The stronger optical density (OD) indicates more platelets have bound.

In ADP activated platelets (Fig. 3.33) GR144053 shows the same amount of negligible binding that it observed in inactive platelets (Fig. 3.32). Activated platelets display a higher OD in comparison to the OD measured in resting platelets, as more platelets will have bound to the fibrinogen coated plate. Sin-1 and GR144053 both significantly decrease platelet adhesion to fibrinogen, with the GpIIb/IIIa inhibitor promoting the most inhibition of binding. The inhibition of binding induced by Sin-1 is likely due to the increase in cGMP that Sin-1 stimulates.

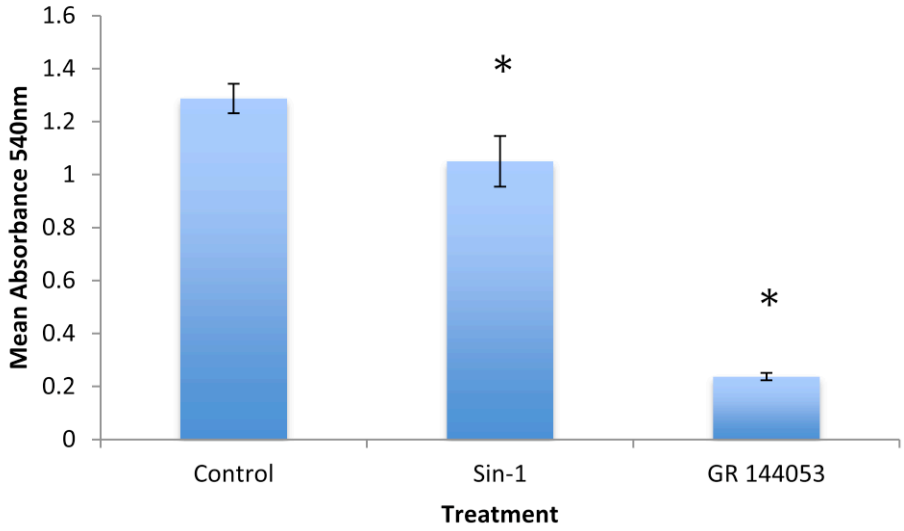


Fig. 3.33- The effect of NO upon ADP activated platelet binding to immobilised fibrinogen. Platelets were either not treated, treated with either Sin-1, or GR 144053, before being added on the fibrinogen coated plate. Statistical analysis calculated to untreated control platelets via paired students t test and indicated via * over respective point, with Sin-1 p = 0.039 and GR 144053 p = 0.008 (n=6). The stronger optical density (OD) indicates more platelets have bound.

3.5 Discussion

ADP induced aggregation requires the simultaneous activation and co-ordination of several signalling cascades. NO can inhibit platelet activation and aggregation by directly increasing intraplatelet cGMP, which is mediated through the binding of NO to the enzyme sGC, catalyzing GTP into cGMP (Naseem and Roberts 2010). This chapter investigated the effect of NO and cGMP upon different platelet functions.

3.5.1 The effect of NO upon ADP induced platelet aggregation

The non enzymatic NO donors Sin-1 and SNP, spontaneously release NO in solution. SNP releases only NO, however this released NO can react with surrounding oxygen creating the superoxide peroxynitrite ($ONOO^-$), Sin-1 however, generates both NO and peroxynitrite. Both NO donors, significantly inhibited ADP induced platelet aggregation (Fig. 3.4). As Sin-1 can also produce $ONOO^-$, to remove any potential effect from the peroxynitrite generated the superoxide scavenging enzyme SOD was utilised (Fig. 3.5). There was no significant difference observed between Sin-1 alone and Sin-1 in the presence of SOD. This indicates that peroxynitrite had no effect on platelet aggregation and that the inhibitory effect observed is entirely due to NO generated by Sin-1. SNP was used as a secondary control, as only NO is generated from this chemical mimetic. The same percentages of inhibition were observed with SNP as with Sin-1, suggesting that both Sin-1 and SNP exert inhibitory effects on platelet aggregation via NO stimulation of platelet cGMP. It is also possible that the released NO from SNP can react with other present radicals to produce a superoxide. As the presence of SOD and Sin-1 displayed no difference it is likely that any produced superoxides were not influencing platelet aggregation and the inhibition obersved is entirely due to NO acting to induce cGMP.

NO enhances platelet cGMP through the direct stimulation of the enzyme soluble guanylyl cyclase. NO binds to haem sites located on sGC, catalyzing GTP into cGMP (Tseng *et al* 2000). The synthesis of cGMP is therefore dependent upon sGC and inhibition of the enzyme will prevent production of cGMP as well as corresponding signalling. If the inhibitory effects of Sin-1 are solely due to increasing intraplatelet cGMP concentrations,

the inhibition of sGC will remove the inhibition induced by Sin-1 on platelet aggregation. The presence of the sGC inhibitor ODQ, removed the inhibition of platelet aggregation that is observed with low concentrations of the NO donor, 8 μM Sin-1 (Fig. 3.6). These results suggest that the inhibition of aggregation induced by 8 μM Sin-1 is cGMP dependent and were confirmed by directly quantifying intraplatelet cGMP after treatment with Sin-1 and ODQ (Fig. 3.13 - 3.15).

In the presence of high concentrations of the NO donor at 100 μM Sin-1, the inhibitory effects of Sin-1 upon aggregation were not fully removed (Fig. 3.7). As sGC is successfully inhibited by the presence of ODQ, this suggests that there is another mechanism in which NO can inhibit platelet aggregation that is independent of cGMP. This observation supports the findings of Crane (2005), Priora (2011) and Wanstall (2007), all of whom obtained similar results, however used other platelet agonists, making the findings in this chapter original. The mechanism by which NO can inhibit platelet activation independent of cGMP is currently under investigation. One proposed mechanism that NO can inhibit platelet activation independent to cGMP is via modification of specific surface receptors. For instance, the nitrosylation of key proteins within the receptors, such as the fibrinogen receptor GPIIb/IIIa, would prevent the binding of necessary ligands therefore inhibiting platelet aggregation (Priora *et al* 2011).

It is possible that the small amount of DMSO present may also impact upon the amount of NO generated by Sin-1, as DMSO has previosuly demonstrated an ability to inhibit RNS mediated damage (Jia *et al* 2010). To fully investigate the effect of Sin-1 and DMSO upon platelets, further investigations using only these two reagents would be necessary and were not part of the investigations in this thesis.

The initiation of platelet aggregation with ADP involves the inhibition of AC by part of the G protein complex (Weber *et al* 1999, Walter and Gambaryan 2009*)*. As AC is naturally inhibited during the ADP induced aggregation process, the presence of the AC inhibitor SQ 22536 will aid the aggregation process (Haslam *et al* 1978). Increasing platelet cAMP by the stimulator PGE_1 potently inhibits platelet aggregation. In the presence of SQ 22536, the AC inhibitor removed the inhibition of aggregation invoked by PGE_1, indicating that the inhibitor is successfully reversing the inhibition induced by PGE_1 (Fig. 3.8).

Inhibiting both AC and sGC, showed no significant effect upon aggregation (Fig. 3.9). In the presence of both inhibitors, SQ 22536 and ODQ the respective stimulators

PGE_1 and Sin-1 failed to cause an effect on platelet aggregation (Fig. 3.10). This result clearly indicates that the AC inhibitor, SQ 22536 and the sGC inhibitor, ODQ are working efficiently and correctly as they are preventing an increase in cAMP and cGMP due to no inhibition of aggregation being observed.

Stimulation of platelet cyclic nucleotides are quickly degraded by platelet PDE, converting the cyclic nucleotides into an inactive metabolite (Feijge *et al* 2004). Intraplatelet PDE mediate the degradation of cyclic nucleotides with platelets containing 3 isoforms of the enzyme, PDE 2, PDE 3A and PDE 5 (Essayan 2001). Both cAMP and cGMP are substrates for PDE 2 and 3A, however PDE 5 is specific for cGMP (Wilson *et al* 2008). There is much speculation with regards to crosstalk or a cross effect between platelet cAMP and cGMP (Francis *et al* 2010). It is postulated that this cross signalling is mediated by cGMP through PDE3A. cGMP stimulates PDE 2, where as PDE 3A is inhibited by cGMP. Both of these PDE can therefore affect cAMP concentrations (Feijge *et al* 2004). Inhibiting platelet PDE with a non-specific inhibitor IBMX, will allow the accumulation of both cyclic nucleotides resulting in the suppression of platelet aggregation. The presence of IBMX with the NO donors Sin-1 and SNP significantly increases the percentage of inhibition of platelet aggregation in comparison to each NO donor alone (Fig. 3.11). IBMX and Sin-1 causes approximately 50 % more inhibition of aggregation than Sin-1 alone, whereas SNP and IBMX inhibition is only increased by approximately 35 %. This difference may be due to the amount of NO produced by each chemical mimetic, as the presence of peroxynitrite produced by Sin-1 was shown not to effect aggregation (Fig. 3.5).

Inhibiting only cGMP specific PDE 5, using the inhibitor Zaprinast causes a small amount of inhibition of platelet aggregation (Fig. 3.12). In combination with Sin-1, a substantial increase in inhibition is observed and at 8 µM, Sin-1 was shown to inhibit aggregation directly through increases in cGMP.

Inhibiting all PDE with IBMX causes more inhibition when stimulated with Sin-1 than with Zaprinast and Sin-1. In the presence of IBMX cAMP will be allowed to accumulate as well as cGMP, therefore an increase in inhibition would be expected, as both cyclic nucleotides will act to inhibit platelet aggregation.

3.5.2 The effect of NO upon intraplatelet cyclic nucleotides

Increases in platelet cyclic nucleotides will potently inhibit platelet aggregation and activation as well as promoting further downstream signalling through protein kinases that will inhibit such important functions as Ca^{2+} release and the rearrangement of the actin cytoskeleton necessary for platelet shape change (Smolenski 2011).

Low concentrations of Sin-1 inhibit platelet aggregation through inducing increases in cGMP (Fig. 3.6). To directly observe the effect upon cGMP, the cyclic nucleotide was quantified after platelets were stimulated with Sin-1. The NO donor significantly increased intraplatelet cGMP irrelevant of the activation status of the platelet or pretreatment with IBMX. The sGC inhibitor successfully reversed the stimulatory effect of Sin-1 upon cGMP corroborating previous aggregation studies (Fig. 3.13 – 3.15). cGMP concentrations as expected were lower without IBMX pretreatment due to the presence of active platelet PDE acting to degrade cGMP. In activated platelets with IBMX, control concentrations of cGMP were increased in comparison to resting platelets. This is an interesting observation, as concentrations of cGMP would be expected to remain unchanged. Whether cGMP has a role in platelet activation is controversially speculated (Siess 2004). There are some claims that cGMP has a role in platelet activation (Siess 2004), however this is widely discredited, as there is more evidence to suggest that cGMP only has an inhibitory role in platelet activation (Li *et al* 2003, Walter and Gambaryan 2004).

Interestingly, the presence of ODQ and ODQ and Sin-1 in activated platelets without IBMX pretreatment completely abolished cGMP concentrations. This is unusual as endogenous concentrations of cGMP should still be present. A decrease in cGMP is expected in the presence of ODQ, however such a significant decrease past the control concentrations of cGMP is not. This could be due to active PDE or experimental error due to quantifying a molecule of such low concentrations.

There is speculation whether cyclic nucleotides can have a cross effect, with concentrations of one nucleotide influencing the opposing molecule. To investigate this, the effect of the cAMP stimulator PGE_1 was established upon platelet cGMP (Fig. 3.16). This experiment was conducted in the presence of IBMX, thus excluding the activity of platelet PDE. PGE_1 did not influence platelet cGMP, suggesting that either cAMP does not influence cGMP or that any cross effect of either nucleotide is mediated by platelet PDE,

corroborating previous studies identifying PDE as a potential mechanism for a cross linking effect between cyclic nucleotides (Fisch *et al* 1995, Smolenski 2011).

Activation of platelet via ADP involves a subsequent decrease in intraplatelet cAMP. Agents that act to increase cAMP, such as prostacyclins inhibit platelet activation and consequentially aggregation (Weber *et al* 1999). Inhibiting AC with an analogue of adenosine SQ 22536 potentiated ADP induced aggregation and can successfully reverse the inhibitory effects mediated by PGE_1 upon platelets (Fig. 3.8, Haslam *et al* 1978). To observe the full stimulatory effects of PGE_1 upon cAMP, intraplatelet concentrations of the cyclic nucleotide were quantified after treatment with SQ 22536 and PGE_1 both in the presence and absence of IBMX.

Resting platelets with IBMX pretreatment displayed the highest concentrations of cAMP, with SQ 22536 preventing the stimulatory effects of PGE_1 upon platelet cAMP (Fig. 3.17). In ADP activated platelets with IBMX, cAMP concentrations were slightly decreased as expected, however SQ 22536 still successfully prevented any increase in cAMP mediated by PGE_1 (Fig 3.18). The absence of IBMX in ADP activated platelets decreased cAMP concentrations further as expected, due to the uninhibited activity of platelet PDE, however SQ 22536 still successfully prevented an increase in cAMP (Fig 3.19).

There is gathering evidence that platelet cyclic nucleotides have a cross effect upon one another, this is debated to be either due to platelet PDE or protein kinases (Smolenski 2011). To establish whether there is an effect between cyclic nucleotides, each was directly quantified after platelets were stimulated with an opposing agent. The effect of the cAMP stimulating PGE_1 upon cGMP was investigated and PGE_1 displayed no effect upon platelet cGMP (Fig. 3.16). It is reported that Sin-1 can increase platelet cAMP whilst in the presence of AC stimulators (Fisch *et al* 1995) and data also suggests that cGMP can influence cAMP via PDE, specifically PDE 3A and PDE 2 (Francis *et al* 2010). The effect of Sin-1 upon cAMP was investigated in the presence and absence of IBMX in platelets in a resting and activated state.

In resting and activated platelets pretreated with IBMX, the combination of PGE_1 and Sin-1 substantially increased intraplatelet cAMP (Fig 3.20, Fig. 3.21). ADP activated platelets with no IBMX as expected overall had decreased cAMP concentrations due to active platelet PDE. Sin-1 alone showed a small increase in cAMP, however combined Sin-

1 and PGE_1 exhibited a significant increase in intraplatelet cAMP (Fig. 3.21). These results add further evidence to suggest that cGMP can increase cAMP and that this cross effect of cyclic nucleotides is mediated through platelet PDE.

Increasing platelet cAMP and cGMP can inhibit platelet aggregation as well as disperse formed platelet aggregates, causing a disaggregatory effect (Puri 1999, May *et al* 1998). Stimulators of both cyclic nucleotides were added to ADP induced pre-aggregated platelets (Fig. 3.23). $PGE_{1,}$ a stimulator of cAMP, caused more disaggregation of platelets than Sin-1. The dispersal of aggregates induced by PGE_1 also occurred at a quicker rate than platelet aggregates treated with Sin-1. Platelets contain approximately ten times higher concentration of cAMP than cGMP (Schwarz *et al* 2001), therefore this may account for difference between the disaggregatory effects of PGE_1 to Sin-1. These results also suggest that cAMP has a more potent inhibitory effect upon platelets than cGMP.

3.5.3 The metabolites of NO in platelets

The metabolism of NO is complex and the direct measurement of NO can be difficult due to the short half-life of the free radical (Guevara *et al* 1998, Sun *et al* 2003). NO metabolites can remain in the vascular system and be converted into biologically active NO when needed (Lundberg and Weitzberg 2005). A marker of NO activity *in vivo* is nitrite, which can be obtained through diet as well as the metabolism of excess NO (Pacher *et al* 2007). To observe platelet NO activity platelet nitrite was quantified using a simple assay. Platelet nitrite in both cell lysate and the media was measured using the Griess assay (Fig. 3.24). Sin-1 produces both NO and peroxynitrite, thus is a positive control and as expected treatment of platelets with Sin-1 increased the presence of nitrite within the lysate and media sample. The lysate sample however cannot be depicted as an accurate representation, as the concentrations quantified are below the detection limit of the assay of 3 μM.

To investigate further another marker of NO activity was quantified, the nitration of tyrosine residues, creating nitrytyrosine. It is proposed that the formation of nitrotyrosine has a role in important signalling processes (Marcondes *et al* 2006). Nitration of tyrosine residues is thought to prevent the phosphorylation of proteins, a well-

recognized signalling transduction mechanism therefore influencing key cellular messages (Olas and Wachowicz 2007).

The methodology was first trialed using nitrated HSA to establish specificity of the antibody and correct antibody dilutions. The antibody was nitrotyrosine specific, therefore platelet lyastes were also examined. Separated inactive platelet proteins were then probed with an anti-nitrotyrosine monoclonal antibody. The molecular weight of the protein band was higher than the expected 67 kDa and interestingly, treatment with Sin-1 did not significantly increase nitrotyrosine expression. The approximate 100 kDa size of the band corresponded to nitrated alpha-actinin (Fig. 3.25), whom others have observed and identified (Nowak and Wachowicz 2002, Marcondes *et al* 2006). The cytoskeletal isoform 1 α-actinin protein is involved in the cross linking of actin networks during the activation and shape change process in platelets. It is proposed that the protein may provide focal contact points during the restructuring process in activation (Tanaka and Itoh 1998). Marcondes *et al* observed a transient and dose dependent response of the nitrated protein alpha actinin, after activation of platelets with thrombin and pretreatment with SNP (Marcondes *et al* 2006). This suggests that the presence of nitrated α-actinin has a key signalling role in the activation and organization of platelet actin.

As Sin-1 did not significantly increase the expression of nitrotyrosine there may be several explanations. The concentration of Sin-1 used was relatively low and therefore may not have induced a detectable increase in expression of nitrotyrosine. It may also be possible that as platelets were inactive and nitrated α-actinin has a role in agonist induced shape change that there is little difference in expression between the two separate platelet samples.

3.5.4 The effect of NO upon platelet shape change and adhesion

Platelet aggregation and activation occur in two separate stages and ultimately lead to platelet shape change. Increases in intraplatelet cAMP and cGMP inhibit platelet ADP aggregation therefore to investigate the effect of increasing intraplatelet cyclic nucleotide concentrations, the shape change profiles of activated platelets were investigated after being challenged with PGE_1 and Sin-1.

Control platelets displayed the normal phases of aggregation, with platelet changing from a discoid shape to amorphous (Fig. 3.26). As expected treatment with Sin-1 shows some activation of platelets across the time period, however no full aggregate is visible (Fig. 3.27). Platelets pretreated with PGE_1 show decreased activation compared to Sin-1, as there are some activated platelets, however most remain in an inactive spherical shape (Fig. 3.28).

The remodeling of the platelet actin cytoskeleton structure is crucial in the process of platelet shape change. The actin cytoskeleton is destroyed during remodeling, with the restructure of the platelet increasing the proportion of polymeric actin, F-actin (Winokur and Hartwig 1995). ADP induced platelet shape change is mediated by Ca^{2+} unlike other agonist induced shape changes that are reliant upon both Rho Kinase and Ca^{2+} signalling (Bauer *et al* 1999, Ohlman *et al* 2000).

To establish the actin cytoskeleton after treatment with Sin-1 and PGE_1, Platelets were labeled with a FITC conjugated monoclonal anti actin antibody and imaged using fluorescent microscopy. Control platelets displayed the stages of platelet shape change, with brighter leading edges indicating the higher concentration of actin present before forming a clot. Platelets treated with Sin-1 and PGE_1 show some activation of platelets, however the majority of the sample remain inactive due to increased intraplatelet cyclic nucleotide concentrations. Increasing platelet cAMP or cGMP causes the inhibition of Ca^{2+} release from internal stores. ADP induced shape change and therefore actin remodeling is Ca^{2+} dependent. Increases in intraplatelet concentrations of either cyclic nucleotide will therefore prohibit ADP induced shape change.

The binding of fibrinogen is a crucial step in the aggregation process to stablise adhering platelets forming an aggregate. The $\alpha_{IIb}\beta_3$ intergrin is present on the surface of platelets in a low affinity form. Activation of platelets with a soluble agonist such as ADP, initiates inside out signalling within the platelet causing a conformational change within $\alpha_{IIb}\beta_3$, thus allowing the binding of soluble fibrinogen and full aggregation of the platelet. The inactive low affinity form, can bind to immobilized fibrinogen triggering outside in signalling through the spreading of the platelet. Such biochemical signals include increases in tyrosine phosphorylation of signalling proteins such as Syk (Src kinases), focal adhesion kinases (FAK) that are important in mediating actin polymerization for platelet shape change (Payrastre *et al* 1999, Rosado *et al* 2000, Cardoso *et al* 2010).

Nitric oxide can mediate platelet functionality through the inhibition of aggregation, granule secretion and adhesion spreading. Platelets treated with Sin-1 displayed a decrease in fibrinogen binding both in resting and ADP activated states (Fig. 3.32, Fig. 3.33). An increase in cGMP via sGC is responsible for the inhibitory effects of NO upon platelets. An increase in cGMP induced by Sin-1 prevents platelet activation and therefore also decreased platelet binding to fibrinogen (Fig. 3.32). High concentrations of NO can inhibit platelet aggregation by mechanism that is independent of cGMP, there is evidence that NO may also inhibit fibrinogen binding via similar mechanism (Cardoso *et al* 2010, Oberprieler *et al* 2007). At 8 μM Sin-1 however, inhibition is mediated directly through increases in cGMP, as the presence of the sGC inhibitor removes any inhibitory effects of Sin-1 on platelets. This indicates that the inhibition of fibrinogen binding by Sin-1 in these experiments is due to Sin-1 acting to increase intraplatelet cGMP.

3.6 Overview of Results:- Key Points

- NO can inhibit platelet aggregation independently of increasing cGMP
- The inhibition of PDE causes inhibition of aggregation and is necessary to fully observe any effects when directly quantifying cyclic nucleotides
- Sin-1 can increase cAMP in presence of AC activators
- PGE_1 does not affect platelet cGMP
- ODQ successfully prevents the inhibition of platelet aggregation and increases in cGMP provoked by 8 µM Sin-1
- SQ 22536 successfully prevents the inhibition of platelet aggregation and increases in cAMP provoked by 10 nM PGE_1
- Platelets challenged with cAMP increasing agents show more disaggregation and inhibition than increases in cGMP stimulators
- Sin-1 inhibits fibrinogen binding at a concentration shown to be cGMP dependent
- The increase in cGMP and cAMP by Sin-1 and PGE_1 respectively, can inhibit ADP induced platelet shape change, including the re-organisation of the actin cytoskeleton

Results from chapter 3 indicate that both cyclic nucleotides have an important inhibitory role in platelet functions and that cGMP can influence cAMP concentrations. Studies also show that the inhibitors used are functioning correctly within the signalling system, allowing specific targets to be isolated.

4 Effect of Aged Garlic Extract on platelet function - *in vitro studies*

4.1 Introduction

Garlic can be found in many preparations from the raw form to a vast array of commercially produced garlic supplements. There is considerable debate as to which preparation of garlic is most beneficial to humans, as each form has different properties (Stanger *et al* 2012, Rahman 2007). One such commercial preparation of garlic is aged garlic extract (AGE) which is marketed in both capsule and liquid forms under the commercial name of Kyolic®, a registered trademark of Wakunaga Ltd. This production of AGE causes an increase in the concentration of sulfur-based and water-soluble compounds the major compound being S-allylcysteine (SAC), which is used to standardize AGE (www.Kyolic.com). Evidence from research over the past 20 years suggests that AGE may reduce some of the risk factors that can contribute and ultimately lead to CVD (Steiner and Li 2001, Campbell *et al* 2001, Allison *et al* 2006a, Rahman and Lowe 2006, Graham *et al* 2012).

One of the major complications of CVD is a thrombotic episode, in which platelets play an important role. The hyper activation of platelets found in CVD is highly associated with the development of thrombotic plaques and these often lead to the occlusion of the vascular vessels resulting in a stroke, myocardial infarction or even death (Projahn and Koenan 2012). There is considerable evidence that AGE has an inhibitory effect upon platelet activation in both healthy subjects and in subjects with CVD (Campbell *et al* 2001, Allison *et al* 2006a, Budoff *et al* 2009).The inhibition of platelets remains an important functional target in the treatment and prevention of cardiovascular episodes. The anti-platelet drugs currently being used for the treatment of CVD have different pharmacokinetics and mechanisms of action in reducing platelet aggregation. For example clopidogrel and ticlopidine target the ADP receptor $P2Y_{12}$ by irreversible binding to the purinergic receptor (Floyd 2012), whilst Aspirin irreversibly inhibits cyclooxgenase (COX), thus stopping the production of thromboxane A_2 (Awtry and Loscalzo 2000, Lordkipanidze 2012). Recently, there has been an increase in the use of alternative therapies such as

medicinal herbs and phytochemicals to help treat CVD. These specific fruits, plants and herbs have attracted much interest and research for their apparent health benefits.

The garlic supplement AGE has been shown to inhibit ADP and collagen induced platelet aggregation both *in vitro* and *in vivo* (Rahman and Billington 2000, Allison 2006b). It is reported that AGE inhibits platelet aggregation by acting upon several signalling mechanisms including intraplatelet cAMP and Ca^{2+} (Allison *et al* 2006a, Allison *et al* 2012), however, the effect of AGE upon platelet cGMP is unknown.

There are studies that indicate there may be a cross over effect by cGMP on cAMP, suggesting that one cyclic nucleotide maybe able to influence the concentrations of the other (Feijge *et al* 2004, Siso-Nadal *et al* 2009). As AGE can increase cAMP, it is therefore possible that AGE may also exert its inhibitory effects on platelets through influencing cGMP. The results of the experiments investigating the effect of AGE on signaling pathways specifically NO and cGMP are reported in this chapter.

4.2 Aims and objectives of the studies in this chapter

The aim of the studies within this chapter was to identify the effect of AGE upon platelet functions and the signalling molecules NO, cGMP and cAMP. This was achieved using the same inhibitors to isolate specific targets that were established to be functioning correctly in Chapter 3.

4.3 Overview of experimental approach

A brief summary of the experiments performed is provided below:

- Aggregation studies investigating the effect of AGE on specific platelet signaling targets (cGMP and cAMP) using the stimulators Sin-1 and PGE_1 and inhibitors ODQ, SQ 22536, IBMX and Zaprinast (2.5, p32)
- Enzyme linked immunoabsorbant assays investigating the effect of AGE upon intraplatelet concentrations of the cyclic nucleotides cAMP and cGMP (2.6, p37)
- Disaggregation of platelet aggregation induced by AGE (2.7.1, p39)

- Fibrinogen Binding Assay to assess the effect of AGE on ADP induced platelet binding to fibrinogen (2.72, p40)
- The effect of AGE on platelet nitrite (2.8, p42)
- SDS PAGE + Western Blot of platelet proteins highlighting the presence of nitrotyrosine in platelets after treatment with Sin-1 and or AGE (2.9, p46)
- SEM imaging of the shape profile of ADP activated platelets after treatment with AGE (2.10.1, p49)
- Fluorescent imaging of platelet actin structure in ADP activated platelets after treatment with AGE (2.10.2, p49)

4.4 Results

4.4.1 The effects of Aged garlic extract upon platelet functions

4.4.1.1 The effect of Aged garlic extract on ADP induced platelet aggregation

PRP (platelet rich plasma) (2.5 x 10^5 platelets / ml) was incubated with varying concentrations of AGE 0.195 % - 25 % (v/v), (final concentrations). A 1 min incubation period proved sufficient to induce inhibition of ADP induced platelet aggregation, which was initiated by the addition 8 µM (final concentration) of the agonist ADP. AGE inhibits ADP platelet aggregation in a dose dependent manner, and each aggregation curve was analysed for total percentage aggregation and the rate of aggregation (Fig. 4.1). Throughout this chapter, reference is made to the primary and secondary phase characteristics of ADP aggregation, these characteristics have previously been explained in detail in Chapter 3 (Fig. 3.1).

AGE at a concentration range of 0.195 % - 0.585 % (v/v) failed to display any significant inhibition of platelet aggregation when compared to the ADP control (Fig.4.1). However, at a concentration range of 0.78 % - 10 % (v/v) AGE significantly inhibited platelet aggregation and the IC_{50} value was determined to be approximately 3 % (v/v) (Fig.4.1a). An example of ADP induced aggregation curve of platelets treated with 3.12 % (v/v) AGE is presented below (Fig. 4.1b).

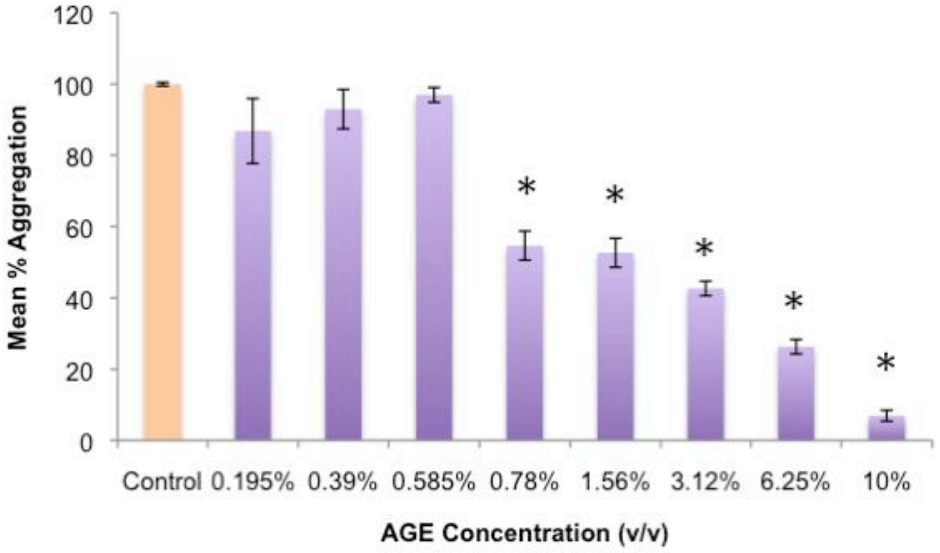


Fig. 4.1a:- The inhibitory effects of AGE upon ADP induced platelet aggregation. Total Percentage aggregation of different concentrations of AGE from 0.195 % to 10 % (v/v) ± SEM. Platelets were treated with differing concentrations of AGE before aggregation was initiated with ADP. Control displays ADP induced platelet aggregation with no AGE present (orange). Points with * indicate statistical significance with $p \leq 0.05$ compared to control data via a paired students t test (n=9).

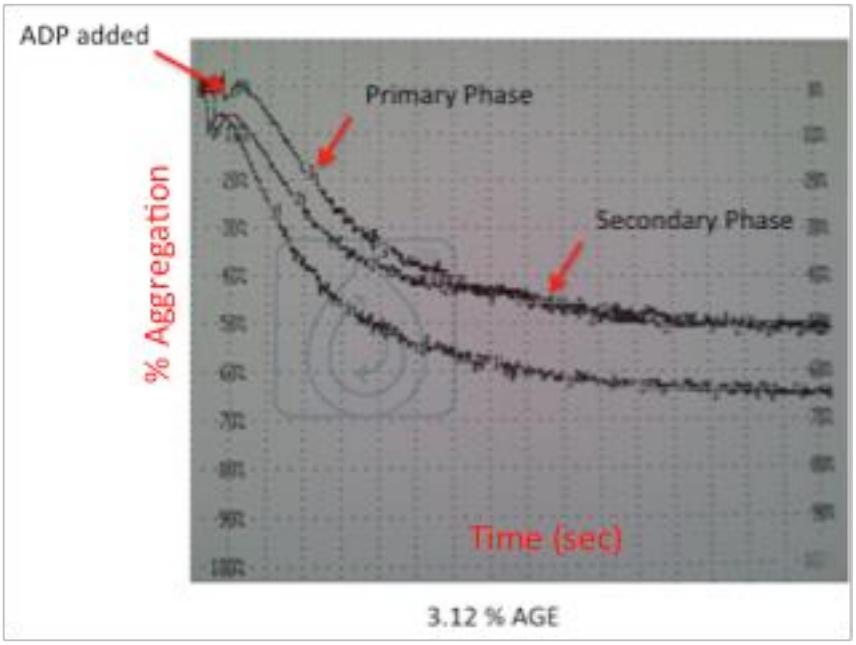


Fig. 4.1b: ADP induced aggregation curve of platelets treated with 3.12 % (v/v) AGE. Aggregation curve showing the inhibition of ADP induced platelet aggregation caused by pretreatment of platelets with 3 12 % AGE (v/v). Activation was initiated by the addition of ADP as indicated, with the primary and secondary phases of aggregation also highlighted.

High concentrations of AGE up to 25 % (v/v) were also investigated and these also significantly inhibited ADP-induced platelet aggregation (Fig.4.2). It was deemed that concentrations of AGE from 10 % (v/v) and above would saturate the system. The dark colour of AGE at such high concentrations could also have interfered with the results, hence AGE was used in further experiments using a concentration range of 0.19 % - 10 % (v/v) (Fig. 4.2).

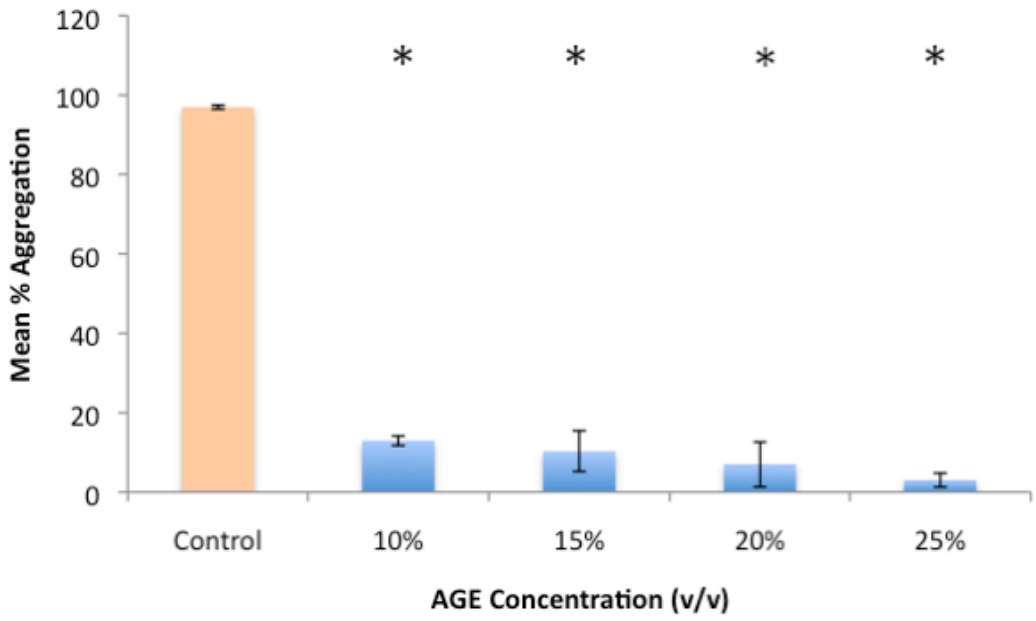


Fig. 4.2:- The inhibitory effects of high concentrations of AGE upon ADP induced platelet aggregation. The total percentage aggregation is presented after platelets were treated with concentrations of AGE between 10 % to 25 % (v/v) ± SEM before ADP initiated aggregation. Control displays ADP induced platelet aggregation with no AGE present (orange). Points with * indicate statistical significance with p = 0.02 compared to control data via a paired students t test (n=6).

4.4.1.2 The effect of AGE and NO upon ADP induced platelet aggregation

Increases in platelet cyclic nucleotides can inhibit platelet aggregation, for instance NO can induce the inhibition of platelet aggregation through an increase in cGMP (Chapter 3, Fig. 3.4. Fig. 3.13), whereas AGE can inhibit ADP induced platelet aggregation through the stimulation of cAMP (Allison *et al* 2006a, Allison *et al* 2012). As AGE can

increase cAMP and NO will increase cGMP, a combination of both NO and AGE should further inhibit platelet aggregation and hence this was investigated.

Platelets treated with Sin-1 reduced aggregation to 62.5 % ± 4.04 % (Fig. 4.3) from the control value of 97 %. Samples treated with SNP also decreased aggregation to 65 % ± 4.2 % from the ADP control value of 97 % (Fig. 4.4).

When AGE was present, both Sin-1 and SNP displayed an increase in inhibition of platelet aggregation at the concentrations tested. Across all concentration of AGE with Sin-1, an increase in inhibition compared to AGE alone was observed (Fig. 4.3) and similar results were observed with SNP (Fig. 4.4).

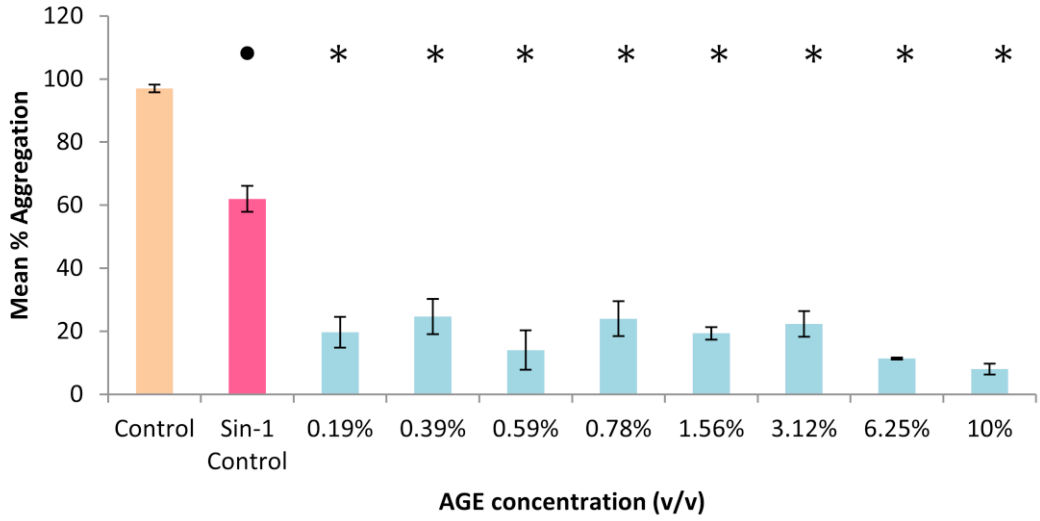


Fig 4.3:- The effect of Sin-1 and AGE upon platelet aggregation. Total Percentage aggregation of AGE plus 8µM Sin-1 ± SEM. Platelets were treated with Sin-1 before further treatment with differing concentrations of AGE from 0.19 % to 10 % (v/v). ADP initiated aggregation, with control data showing aggregation achieved from ADP alone (orange) and Sin-1 alone (pink). Statistically significant data indicated by * above respective points and was calculated to the control Sin-1 data with no AGE present, whereas • indicates significance to the ADP control data. Data was analysed using a paired students t test with $p \leq 0.05$ considered significant (n=9).

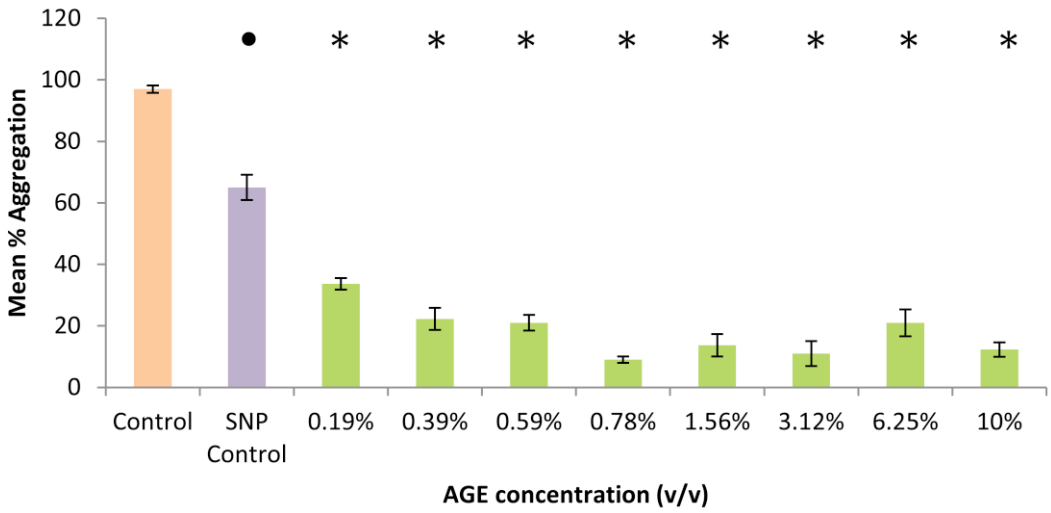


Fig 4.4:- The effect of SNP and AGE upon platelet aggregation. Total percentage aggregation of AGE plus SNP ± SEM. Platelets were treated with SNP before further treatment with differing concentrations of AGE from 0.19 % to 10 % (v/v). Aggregation was initiated via the addition of ADP with control data for ADP alone (orange) and SNP aggregation alone (purple). Statistically significant data indicated by * above respective points and was calculated to the control SNP data, whereas • indicates significance to ADP control data via a paired students t test with $p \leq 0.05$ (n=9).

Sin-1 and SNP both displayed increased inhibition of platelet aggregation in the presence of AGE even at low concentrations of the garlic extract. The increase in inhibition observed in the presence of AGE is statistically significant when compared to the respective NO donor control (Fig. 4.3, Fig. 4.4).

Data is also statistically significant when comparing AGE alone, AGE plus Sin-1 and AGE plus SNP at each individual AGE concentration (Fig. 4.5). The IC_{50} value of AGE was significantly decreased in the presence of both NO donors, decreasing to less than 0.1 % (v/v) compared to IC_{50} value of 3 % (v/v) when using AGE on its own.

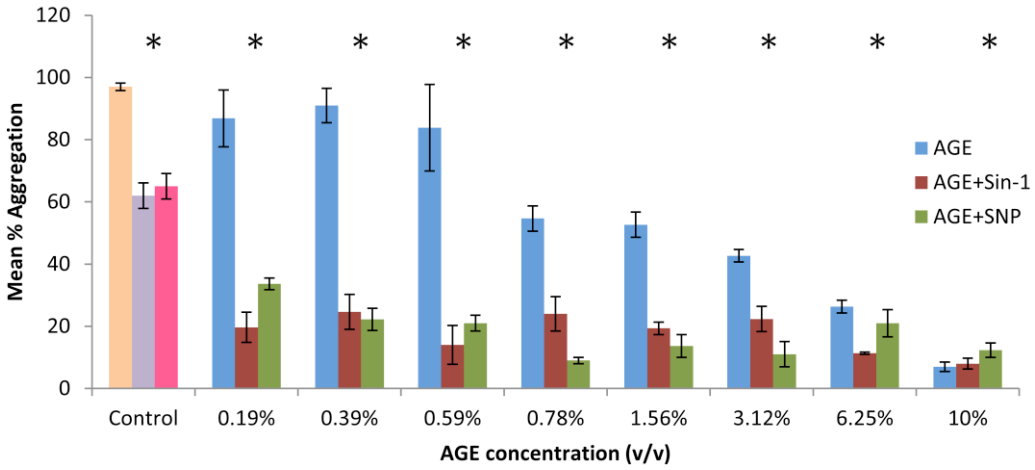


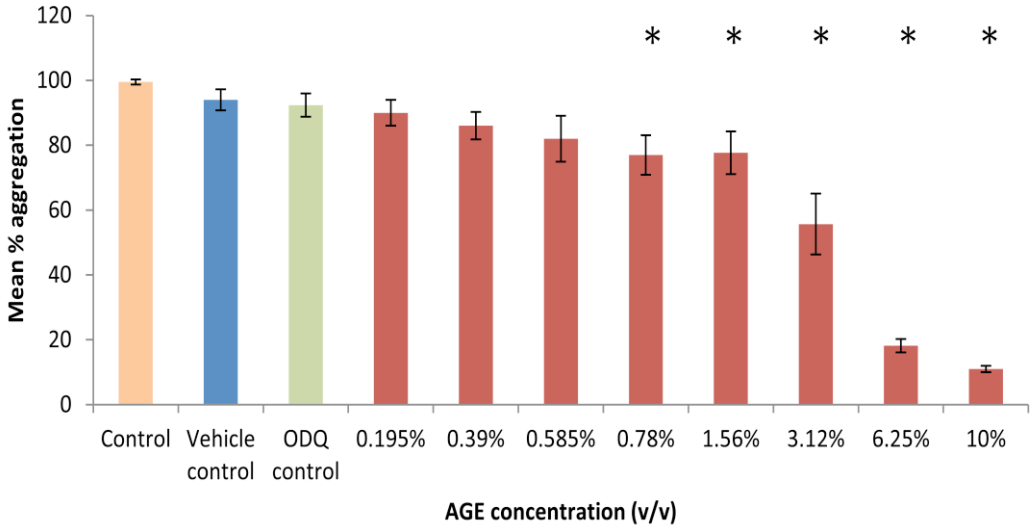
Fig 4.5:- A comparison of the percentage of platelet aggregation in the presence of AGE and the NO donors Sin-1 and SNP. Percentage aggregation of AGE (blue), AGE plus SNP (green) AGE plus Sin-1 (red) ± SEM. Platelets were treated with either PBS, Sin-1 or SNP before further treatment with differing concentrations of AGE from 0.19 % to 10 % (v/v). Aggregation was initiated by the addition of ADP. Control data for ADP aggregation alone (orange), SNP (pink) and Sin-1 (purple) without AGE are highlighted. Statistically significant data indicated by * above respective points and was calculated via oneway ANOVA across data per AGE concentration with $p \leq 0.05$ (n=9).

4.4.1.3 The effect of AGE upon soluble guanylyl cyclase

Inhibiting sGC should remove any inhibitory effect of Sin-1 upon platelet aggregation. The inhibitor 1*H*-[1,2,4] Oxadiazolo [4,3-*a*] quinoxalin-1-one (ODQ) selectively and irreverisibly binds to the haem site of sGC, blocking the binding of NO and therefore the transduction of NO signaling via sGC. The effect of inhibiting sGC and the effect of AGE upon NO mediated inhibition of platelet aggregation was thus investigated.

The sGC inhibitor ODQ was reconstituted in DMSO and further diluted using PBS to ensure that no more than 0.5 % DMSO was present. The vehicle control utilised an equal dilution of DMSO to ensure there was no interference upon platelet aggregation, this was true for all experiments which utilised DMSO as a vehicle control. A minimal percent of inhibition of platelet aggregation is observed by the vehicle control decreasing to 94 % ± 2.55 %, when compared to the ADP control percentage of aggregation of 97 % ±

1.2 %, however this decrease is not significant. ODQ alone induced a small amount of inhibition of aggregation decreasing to 90 % ± 2.08 %, however this is not significant to the relative vehicle control of 94 % ± 2.55 % (Fig. 4.6a).



Platelets were treated with ODQ before further treatment with AGE with concentrations ranging from 0.19 % - 10 % (v/v). Aggregation was initiated by the addition of ADP. ODQ (green) and vehicle control (blue) show the small amount of inhibition achieved with ODQ alone and the diluted DMSO compared to standard ADP aggregation (orange). Statistical analysis performed to ODQ control and indicated by * above respective point with p = 0.04 via a paired students t test (n=6).

In the presence of ODQ, AGE displayed varying results, when compared to the data obtained with AGE on its own (Fig. 4.6b). The concentrations 0.195 %, 0.39 %, 0.585 %, 1.56 % and 10 % (v/v) of AGE respectively indicated no difference with the addition of ODQ. At 0.78 % and 1.56 % (v/v) of AGE, ODQ partially reversed the inhibitory effects of AGE when compared to AGE alone. At 6.25 % (v/v) of AGE the presence of ODQ acted to increase the inhibition of platelet aggregation in comparison to AGE alone (Fig. 4.6b).

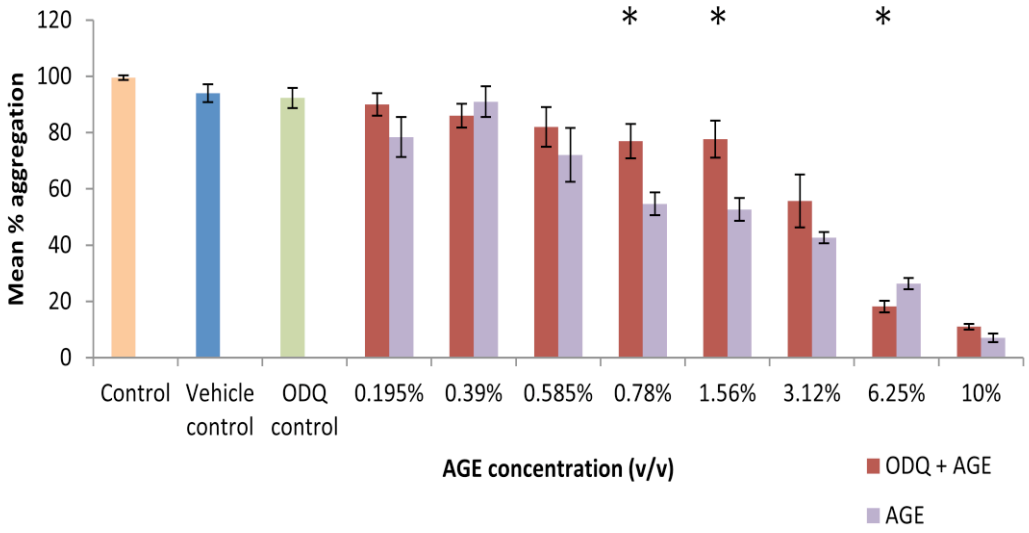


Fig. 4.6b – A comparison of the percentage of platelet aggregation in the presence of AGE against AGE and ODQ. Platelets were treated with ODQ before further treatment with AGE with concentrations ranging from 0.19 % - 10 % (v/v). Aggregation was initiated by ADP with ODQ (green) and the vehicle control (blue) showing the small amount of inhibition achieved with ODQ alone and the diluted DMSO vehicle control compared to standard ADP aggregation (orange). Statistical analysis was performed at each AGE concentration between ODQ and AGE and AGE alone data, significance is indicated by * above respective point with p = 0.039 via a paired students t test (n=6).

To fully investigate the effects of ODQ and AGE upon ADP induced platelet aggregation, the rates of aggregation were also determined. ADP aggregation characteristically has two phases of aggregation, therefore each aggregation curve was analysed for any effects upon both the primary and secondary phase aggregation rates (Fig. 4.6c, Table 4.1, Table 4.2).

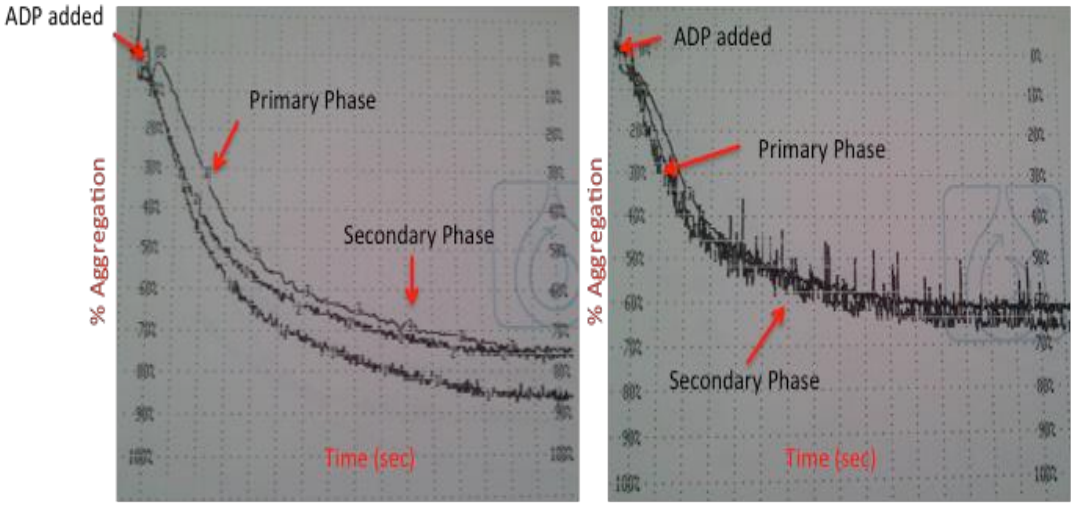


Fig. 4.6c: - Aggregation plot of ADP induced platelet aggregation with ODQ against the aggregation plot of ODQ plus 1.56 % AGE (v/v). Arrows indicate the point at which platelet activation was initiated by ADP as well as the primary and secondary phases of ADP aggregation.

ODQ decreased the primary rate of aggregation to 0.93 ± 0.07 % compared to the control rate of 1.16 ± 0.19 %, however this decrease was not statistically significant. There was no difference in the secondary rates of aggregation between the control and ODQ control (Table 4.1).

Primary Rates of ADP induced Aggregation					
AGE Concentration	**AGE**	**SEM**	**ODQ**	**SEM**	**P value**
(v/v)	% / sec		% / sec		
Control	1.16	0.19	0.93	0.07	0.07
0.19	0.78	0.04	0.9	0.04	0.09
0.39	0.77	0.03	1	0.04	0.12
0.58	0.87	0.05	0.9	0.04	0.11
0.78	0.66	0.08	0.66	0.18	0.06
1.56	0.5	0.03	0.66	0.09	0.06
3.12	0.66	0.06	0.62	0.06	0.35
6.25	0.6	0.07	0.8	0.09	0.22
10	0.2	0.03	0.38	0.03	0.01*

Table 4.1:- The effect of ODQ and AGE on the primary rate of ADP induced aggregation. Average rates of ADP induced platelet aggregation after treatment with AGE in the presence and absence of the sGC inhibitor ODQ. Data is presented as aggregation % / second ± SEM. P values were calculated using a paired students t test between AGE and AGE plus ODQ data with $p \leq 0.05$ considered significant and are highlighted with * (n=6).

In the presence of ODQ the only significant difference in the primary rates between AGE alone and AGE plus ODQ, was obtained at 10 % (v/v) AGE with $p \leq 0.05$ (Table 4.1). In contrast, when the secondary rates of aggregation between AGE alone and AGE plus ODQ, were compared the only significant difference was found at 0.58 % (v/v) AGE with $p \leq 0.05$ (Table 4.2).

Secondary Rates of ADP induced Aggregation					
AGE Concentration	**AGE**	**SEM**	**ODQ**	**SEM**	**P value**
(v/v)	% / sec		% / sec		
Control	0.24	0.05	0.23	0.03	0.14
0.19	0.15	0.05	0.15	0.02	0.11
0.39	0.22	0.04	0.23	0.01	0.44
0.58	0.44	0.05	0.26	0.05	0.001 *
0.78	0.17	0.02	0.26	0.06	0.32
1.56	0.18	0.02	0.23	0.02	0.26
3.12	0.15	0.03	0.15	0.02	0.07
6.25	0.28	0.03	0.48	0.10	0.47
10	0.26	0.03	0.38	0.08	0.17

Table 4.2:- The effect of ODQ and AGE on the secondary rates of ADP induced aggregation. Average rates of ADP induced platelet aggregation after treatment with AGE in the presence and absence of the sGC inhibitor ODQ. Data is presented as aggregation % / second ± SEM. P values were calculated using a paired students t test between AGE and AGE plus ODQ data, with $p \leq 0.05$ considered significant and indicated by * (n=6).

4.4.1.4 The effect of AGE upon adenylyl cyclase

After investigating the effect of AGE upon sGC, its effect on the enzyme Adenylyl cyclase (AC) was established as it has been previously reported that AGE can increase intraplatelet cAMP (Allison *et al* 2012). If AGE inhibits platelet aggregation through only moderating platelet cAMP, inhibiting AC with SQ 22536 should reverse the effects of AGE on aggregation. PRP was first incubated with the AC inhibitor SQ 22536, followed by incubation with differing final concentrations of AGE from 0.19 % to 10 % (v/v). Aggregation was initiated by the addition of ADP (Fig.4.7).

Using the inhibitor of AC, SQ 22536 an increase aggregation was observed as expected. A pro-aggregatory response is evident, as platelet aggregation was increased from a control value with no inhibitor present of 97 % ± 1.2 % to 113 % ± 0.8 %. The AC inhibitor SQ 22536 was reconstituted in $_{d}H_2O$ therefore the vehicle for this inhibitor will have no effect upon aggregation. The presence of AGE and SQ 22536 at all concentrations tested significantly decreased aggregation in comparison to the SQ 22536 control

(Fig.4.7a). Despite this, SQ 22536 did not significantly reverse the inhibition of aggregation caused by AGE across the differing concentrations. This is evident when comparing AGE alone data to SQ 22536 and AGE data (Fig. 4.7b).

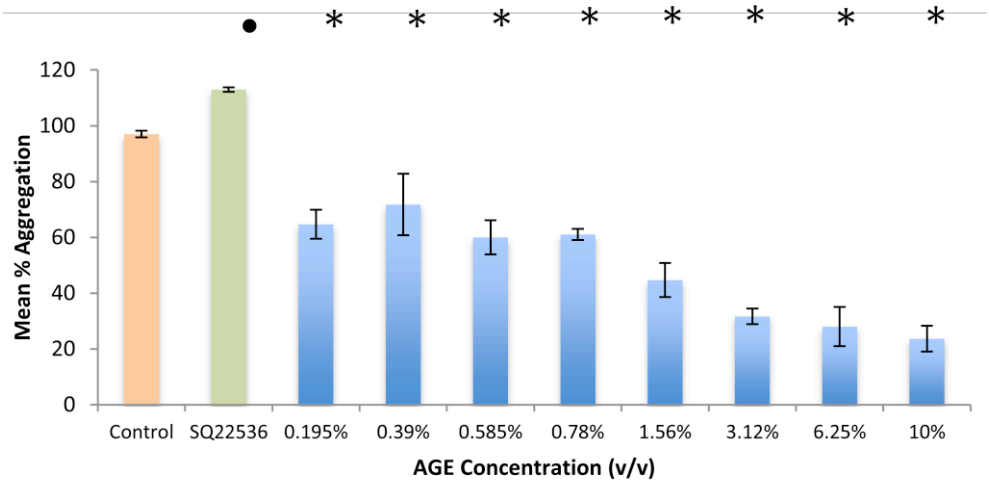


Fig. 4.7a- Percentage aggregation induced by differing AGE concentrations in the presence of SQ 22536. Platelets were treated with SQ 22536 before further treatment with AGE with concentrations ranging from 0.19 % - 10 % (v/v). SQ 22536 (green) shows the significant increase in aggregation in the presence of SQ 22536 alone compared to standard ADP aggregation (orange) and is indicated by • above the respective data with p = 0.03. Statistical analysis was performed against the SQ 22536 control (green) and is indicated by * above respective point with values of p = 0.027 via paired students t test (n=6).

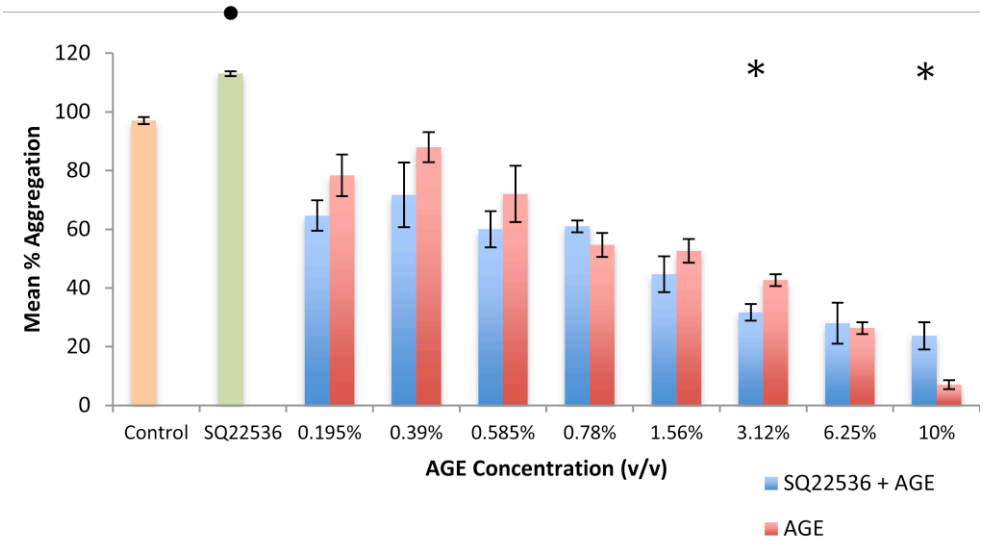


Fig. 4.7b – A comparison of the percentage of platelet aggregation in the presence of AGE and SQ 22536 against AGE. Platelets were treated with SQ 22536 before further treatment with AGE with concentrations ranging from 0.19 % - 10 % (v/v). SQ 22536 (green) indicates the significantly increased aggregation in the presence of SQ 22536 compared to standard ADP aggregation (orange). Statistical analysis performed to SQ 22536 control and indicated by * above respective point with p = 0.029, whereas • indicates significance to the ADP control, with p = 0.03 via paired students t test (n=6).

SQ22536, does however appear to reverse some of the inhibition of aggregation that AGE alone induces at 10% (v/v). This is not a consistent observation, as at 3.12 %, SQ 22536 and AGE appear to cause more inhibition of aggregation compared to AGE alone data (Fig.4.7b). To examine this further, the rates of aggregation were analysed in the presence of the AC inhibitor SQ 22536 (Fig. 4.7c).

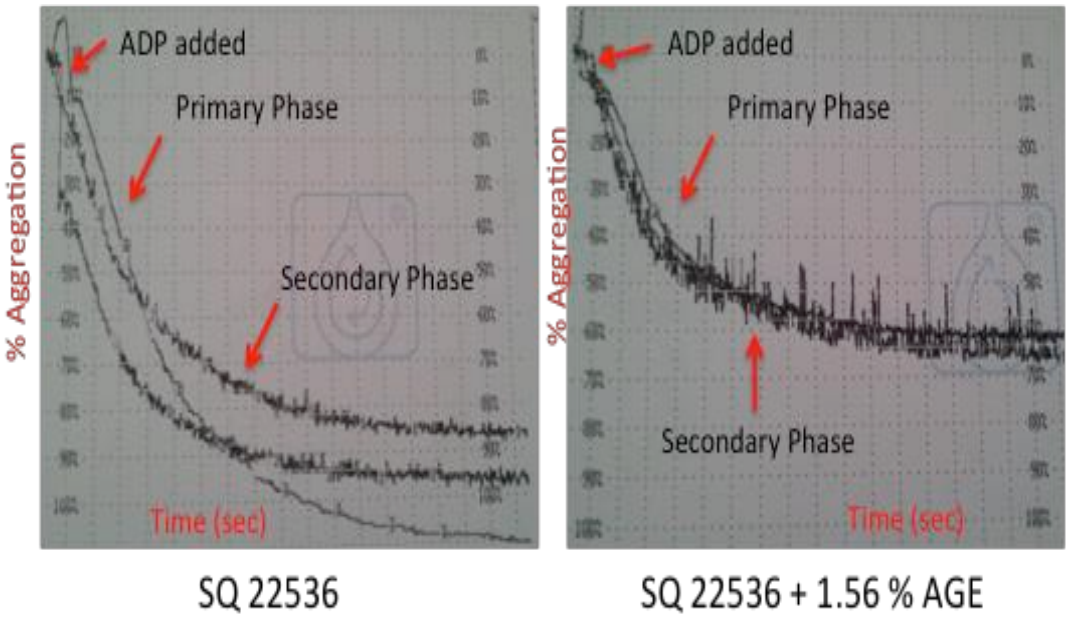


Fig. 4.7c: - Aggregation plot of ADP induced platelet aggregation with SQ 22536 against the aggregation plot of SQ 22536 plus 1.56 % (v/v) AGE. Arrows indicate the addition of ADP to initiate platelet activation as well as the two phases of ADP induced aggregation.

The primary rate of ADP induced platelet aggregation of 1.16 ± 0.19 % / sec was significantly increased in the presence of SQ 22536 to 1.42 ± 0.23 % / sec. After challenging with AGE in the presence of SQ 22536, the primary rate of aggregation was also significantly increased at the concentrations 0.78, 1.56, 3.12, 10 % (v/v) of AGE compared to the primary aggregation rates of AGE alone (Table 4.3).

Primary Rates of ADP induced Aggregation					
AGE Concentration	**AGE**	**SEM**	**SQ 22536**	**SEM**	**P value**
(v/v)	% / sec		% / sec		
Control	1.16	0.19	1.42	0.23	0.01 *
0.19	0.78	0.04	0.69	0.13	0.23
0.39	0.77	0.03	0.53	0.06	0.06
0.58	0.87	0.05	0.42	0.14	0.15
0.78	0.66	0.08	0.8	0.05	0.05 *
1.56	0.5	0.03	0.66	0.10	0.03 *
3.12	0.66	0.06	0.73	0.11	0.04 *
6.25	0.6	0.07	0.46	0.04	0.07
10	0.2	0.03	0.44	0.06	0.003 *

Table 4.3:- The effect of SQ 22536 on the primary rate of ADP induced platelet aggregation. Average rates of ADP induced platelet aggregation after treatment with AGE in the presence and absence of the AC inhibitor SQ 22536. Data is presented as aggregation % / second ± SEM. P values were calculated using a paired students t test between AGE and SQ 22536 plus AGE data with $p \leq 0.05$ considered significant and highlighted with * (n=6).

The secondary rates of aggregation were significantly decreased at 0.58 % and 10 % (v/v) AGE in the presence of SQ 22536 (Table 4.4).

Secondary Rates of ADP induced Aggregation					
AGE Concentration	**AGE**	**SEM**	**SQ 22536**	**SEM**	**P value**
(v/v)	% / sec		% / sec		
Control	0.24	0.05	0.16	0.16	0.26
0.19	0.15	0.05	0.11	0.02	0.09
0.39	0.22	0.04	0.17	0.02	0.11
0.58	0.44	0.05	0.1	0.03	0.02 *
0.78	0.17	0.02	0.2	0.02	0.19
1.56	0.18	0.02	0.21	0.03	0.08
3.12	0.15	0.03	0.15	0.01	0.14
6.25	0.28	0.03	0.26	0.05	0.12
10	0.26	0.03	0.18	0.04	0.003 *

Table 4.4:- The effect of SQ 22536 on the secondary rates of ADP induced platelet aggregation. Average rates of ADP induced platelet aggregation after treatment with AGE in the presence and absence of the AC inhibitor SQ 22536. Data is presented as aggregation % / second ± SEM. P values were calculated using a paired students t test between AGE and AGE plus SQ 22536 data, with $p \leq 0.05$ considered significant and highlighted with * (n=6).

4.4.1.5 The effect of AGE upon platelet aggregation when soluble guanylyl cyclase and adenylyl cyclase are inhibited

Inhibiting either AC or sGC singularly did not fully reverse the inhibitory effects of AGE upon platelet aggregation. Using both ODQ and SQ 22536 the effect of AGE upon platelet aggregation was hence investigated. This will help elucidate whether the inhibitory actions of AGE acts soley through cyclic nucleotide signalling (Fig.4.8).

The sGC inhibitor, ODQ was reconstituted in DMSO, and further diluted using PBS to ensure a relatively low amount of DMSO was present, whereas the AC inhibitor SQ22536 was reconstituted in $_{d}H_2O$ and therefore had no effect upon platelet aggregation. A 3 % inhibition of platelet aggregation was observed in the presence of SQ 22536 when compared to the control data, however this was not statistically significant.

When combined with both inhibitors, AGE at all concentrations significantly inhibited ADP platelet aggregation (Fig.4.8a).

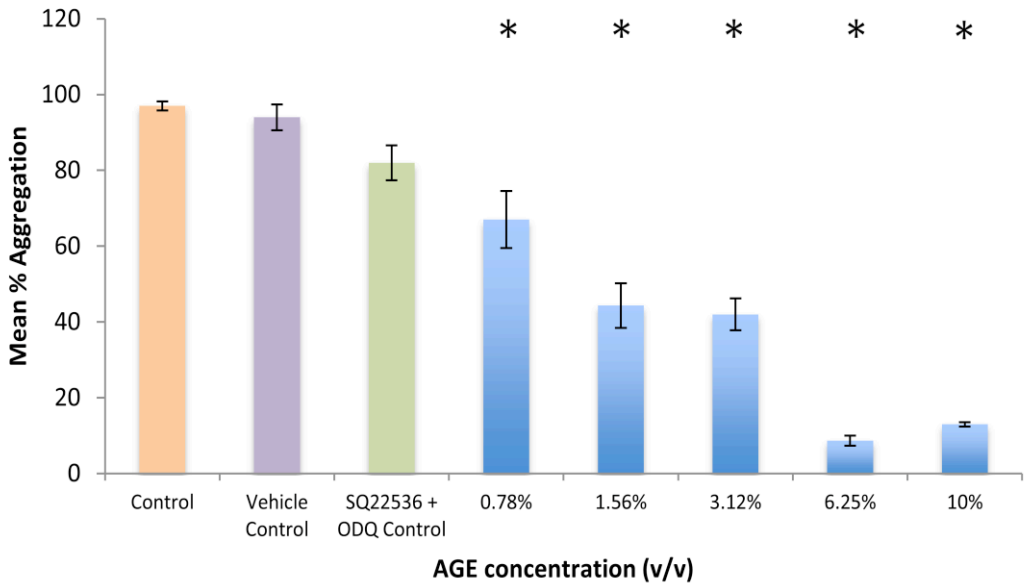


Fig. 4.8a:- Percentage aggregation induced by differing AGE concentrations in the presence of SQ 22536 and ODQ. SQ 22536 and ODQ (green) and vehicle control (purple) show the small amount of inhibition achieved with SQ 22536 and ODQ alone and the diluted DMSO compared to standard ADP aggregation (orange). Statistical analysis performed to SQ 22536 + ODQ control and indicated by * above respective point with values of p = 0.02 via paired students t test (n=6).

At 6.25 % (v/v) AGE, the presence of the inhibitors act to increase the inhibition of platelet aggregation, when compared to AGE with no inhibitors present (Fig 4.8b). In contrast at 10 % (v/v) AGE, the opposite result is observed. The presence of ODQ and SQ 22536 appears to reverse the inhibitory effects normally observed at 10 % (v/v) AGE. Only at the concentrations 6.25 % and 10 % (v/v) of AGE does the presence of the inhibitors cause a significant difference (Fig 4.8b).

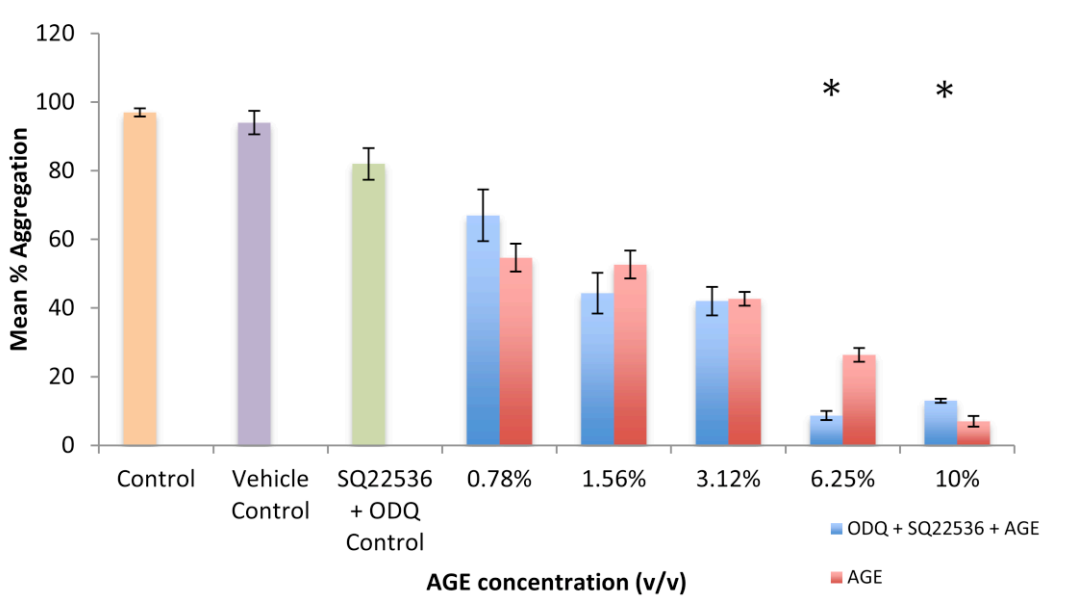


Fig. 4.8b:- Comparison of differing AGE concentrations (v/v) in the presence of SQ 22536 and ODQ and AGE alone on platelet aggregation. SQ 22536 and ODQ (green) and vehicle control (purple) show the small amount of inhibition achieved with SQ 22536 and ODQ alone and the diluted DMSO compared to standard ADP aggregation (orange). Statistical analysis performed at each AGE concentration between AGE alone and AGE + ODQ + SQ 22536 data and is indicated by * above respective point with p = 0.02 via a paired students t test (n=6).

As opposing results were obtained, the rates of aggregation of AGE alone and AGE plus SQ 22536 and ODQ were also analysed.

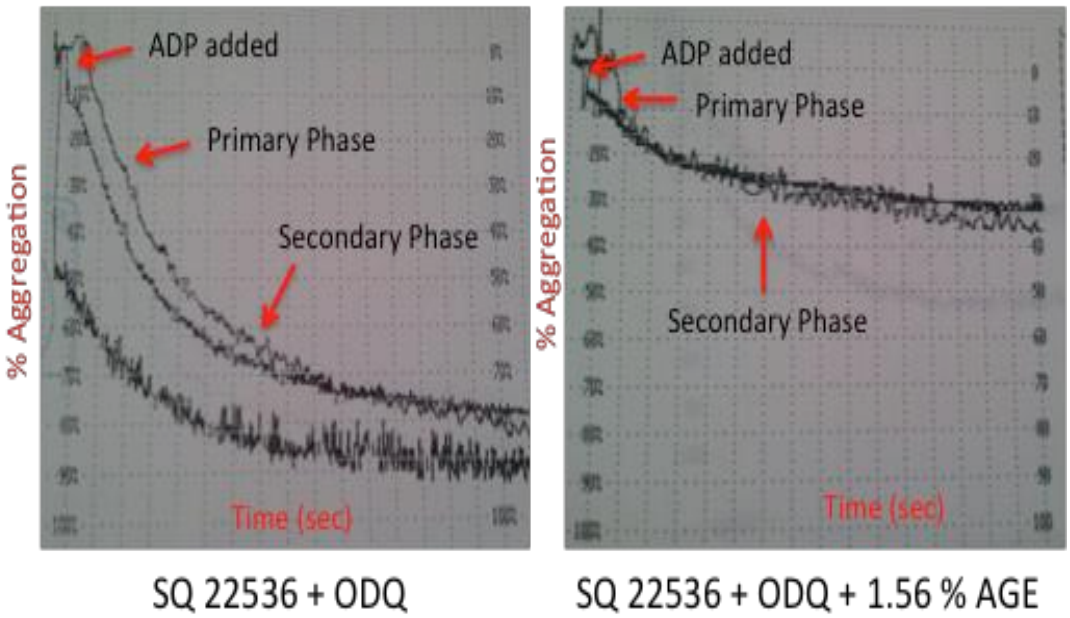


Fig. 4.8c: - Aggregation plot of ADP induced platelet aggregation with ODQ and SQ 22536 against the aggregation plot of SQ 22536 and ODQ plus 1.56 % (v/v) AGE. Activation was initiated by the addition of ADP as indicated. The primary and secondary phases of aggregation are also indicated.

The primary rate of aggregation was seen to increase in the presence of the AC inhibitor, SQ 22536 to 1.4 ± 0.23 % / sec against the control rate of 1.16 ± 0.19 % / sec. ODQ alone exhibited a decrease in the primary rate to 0.93 ± 0.07 % / sec, however in the presence of both inhibitors SQ 22536 and ODQ the rate increased (1.33 ± 0.15 % / sec) (Table 4.5).

Primary Rates of ADP induced Aggregation					
AGE Concentration	**AGE**	**SEM**	**SQ 22536 + ODQ**	**SEM**	**P value**
(v/v)	% / sec		% / sec		
Control	1.16	0.19	1.33	0.15	0.14
0.78	0.66	0.08	1.02	0.04	0.02 *
1.56	0.5	0.03	0.65	0.09	0.07
3.12	0.66	0.06	0.75	0.12	0.49
6.25	0.6	0.07	0.5	0.13	0.15
10	0.2	0.03	0.53	0.11	0.39

Table 4.5:- The effect of inhibitors on the primary rate of ADP induced aggregation. Average rates of ADP induced platelet aggregation in the presence and absence of both sGC and AC inhibitors ODQ and SQ 22536 respectively. Data is presented as aggregation % / second ± SEM. P values were calculated between AGE and SQ 22536, + ODQ + AGE data, using a paired students t test with $p \leq 0.05$ considered significant and are indicated with * (n=6).

In the presence of ODQ and SQ 22536, the only significant difference in the rates of aggregation was observed at 0.78 % (v/v) of AGE. The presence of the inhibitors acted to increase both the primary and secondary rates of aggregation (Table 4.5 and 4.6).

Secondary Rates of ADP induced Aggregation					
AGE Concentration	**AGE**	**SEM**	**SQ 22536 + ODQ**	**SEM**	**P value**
(v/v)	% / sec		% / sec		
Control	0.24	0.05	0.25	0.03	0.31
0.78	0.17	0.08	0.26	0.02	0.04 *
1.56	0.18	0.03	0.18	0.02	0.23
3.12	0.15	0.06	0.15	0.01	0.12
6.25	0.28	0.07	0.57	0.11	0.34
10	0.26	0.03	0.13	0.07	0.41

Table 4.6:- The effect of inhibitors on the secondary rates of ADP induced aggregation. Average rates of ADP induced platelet aggregation in the presence and absence of both sGC and AC inhibitors ODQ and SQ 22536 respectively. Data is presented as aggregation % / second ± SEM. P values were calculated between AGE and SQ 22536, + ODQ + AGE data, using a paired students t test with $p \leq 0.05$ considered significant and indicated by * (n=6).

Interestingly when studying the total percentage of aggregation significant results with AGE and both inhibitors were obserevd at high concentrations of AGE namely 6.25 % and 10 % AGE (v/v). However, in contrast to this the rates of aggregation were only significant at 0.78 % (v/v) AGE.

4.4.1.6 Effect of AGE on platelet aggregation in the presence of PDE inhibitors

AGE can influence platelet cAMP (Allison *et al* 2006) and possibly cGMP. It is therefore of interest to investigate the effect upon platelet aggregation when platelet PDE is inhibited, as PDE mediate platelet cAMP and cGMP concentrations. If AGE exerts inhibitory effects on platelets through the stimulation of cyclic nucleotides, an increase in the inhibition of aggregation would be observed in the presence of a PDE inhibitor.

Isolated PRP was first incubated with 100 µM (final concentration) of IBMX for 10 minutes to allow full permeation of the inhibitor into the platelet. This concentration and time period has previously been shown to be effective at inhibiting platelet PDE (Burrell *et al* 2008). Following treatment with IBMX, platelets were further challenged with final concentrations of AGE ranging from 0.19 % to 10 % (v/v) and ADP initiated aggregation.

To ensure that the DMSO vehicle had no effect upon aggregation, a control was investigated utilising the same dillution of DMSO in which IBMX was reconstituted and subsequently diluted. The vehicle control showed little effect upon aggregation decreasing aggregation to 94 % ± 2.55 % from 97 % ± 1.2 % of the ADP control. IBMX alone significantly decreased platelet aggregation by a total of 31 %, $p \leq 0.05$ when compared to the vehicle control (Fig. 4.9).

Across all concentrations of AGE from 0.19 % to 10 % (v/v) the presence of IBMX substantially inhibited platelet aggregation when compared to AGE alone at the same concentration. The decrease in aggregation in the presence of IBMX was statistically significant at each concentration of AGE, when compared against AGE alone (Fig. 4.9).

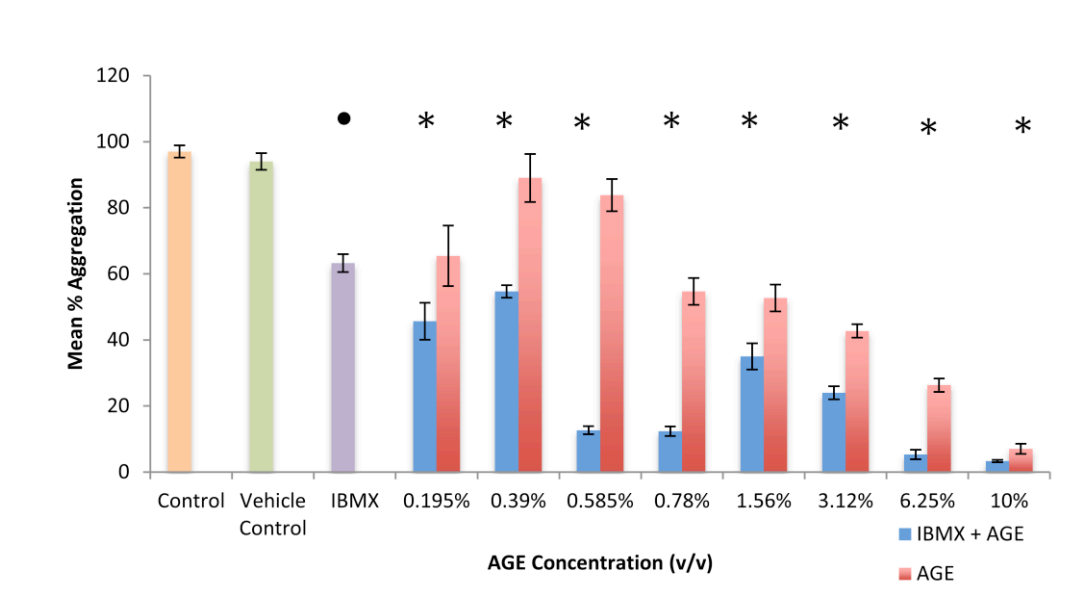


Fig. 4.9:- The effect of IBMX and AGE upon platelet aggregation. Percentage platelet aggregation ± SEM after treatment with IBMX and AGE (blue) compared to platelets incubated with AGE alone (red) against the three controls of ADP aggregation (orange), IBMX alone (green), and the vehicle control DMSO (purple). Statistically significant points are marked with * above the respective point $p \leq 0.05$. Statistics were calculated by comparison of AGE (red) to IBMX and AGE (blue) at each AGE concentration using a paired students t test. • indicates significance to vehicle control data with $p = 0.032$ (n=9).

In order to investigate the effect of AGE on cGMP a specific inhibitor Zaprinast was used. Zaprinast is a specific inhibitor for the cGMP phosphodiesterase PDE5 and was utilised in combination with AGE in aggregation experiments. If AGE inhibits platelet aggregation through cGMP influenced signaling, inhibiting PDE5 which is cGMP specific will cause a substantial increase in inhibition of aggregation. Inhibiting PDE5, will cause an increase inhibition of aggregation due to the accumulation of intraplatelet cGMP (Holbrook and Coker 1991).

PRP was first incubated with Zaprinast before being challenged with final concentrations of AGE ranging from 0.78 % to 10 % (v/v) (Fig.4.10).

Zaprinast was reconstituted in DMSO therefore an appropriate vehicle control DMSO ≤ 0.5 % was utilised. This vehicle control decreased the total percentage of aggregation to 94 % ± 2.55 %, from the ADP control value of 97 % ± 2.55 %, however this is not statistically significant. Zaprinast alone inhibited aggregation to 86 % ± 3.05 % from 94 % ± 2.55 % of the vehicle control and this was statistically significant. The presence of

Zaprinast with AGE caused significantly more inhibition of aggregation at concentrations from 0.78 % to 6.25 % (v/v) of AGE than AGE alone achieved. At 10 % (v/v) AGE, there was no difference between AGE alone and AGE plus Zaprinast as both showed maximum inhibition of platelet aggregation. The combination of AGE and Zaprinast significantly increased inhibition of ADP induced platelet aggregation (Fig. 4.10).

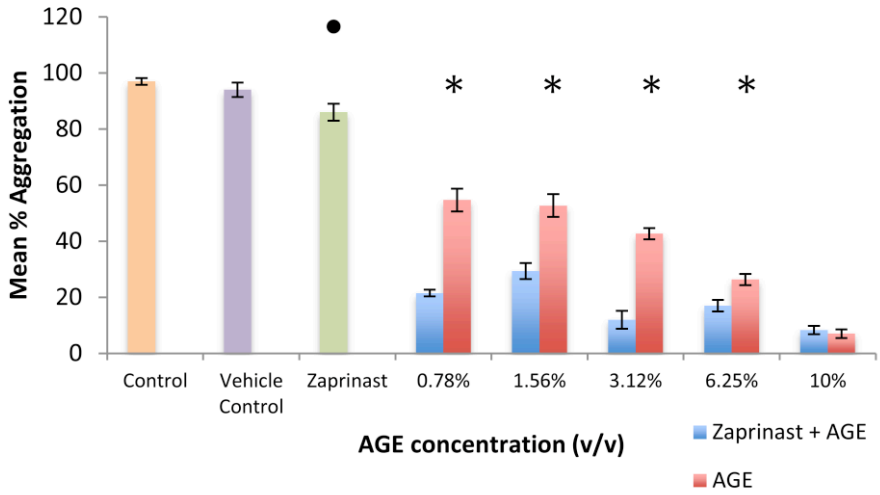


Fig. 4.10:- The effect of Zaprinast and AGE on platelet aggregation. Percentage platelet aggregation ± SEM of Zaprinast plus AGE (blue), and AGE alone (red) against three controls, ADP control (orange), DMSO vehicle control (purple) and Zaprinast (green). Statistically significant points are marked with * above the respective points with p values of 0.028. Statistical significance was calculated by comparison of Zaprinast (green) to Zaprinast plus AGE (blue) at each AGE concentration using a paired students t test. • indicates significance to vehicle control (purple) data with p = 0.04 (n=6).

Data obtained using both PDE inhibitors IBMX and Zaprinast show that AGE definitively targets intraplatelet cyclic nucleotides. As the effect of AGE upon intraplatelet cGMP is unknown and aggregation studies indicate cGMP may be a target of AGE, this cyclic nucleotide was directly quantified after platelets were treated with AGE.

4.4.2 The effect of AGE upon platelet cGMP

4.4.2.1 The effect of 5 % AGE and ODQ upon intraplatelet cGMP concentrations

To investigate the effect of AGE upon cGMP, intraplatelet cGMP was directly quantified after being challenged with different concentrations of AGE. As intraplatelet cGMP is present in low concentrations, platelets were first treated with the PDE inhibitor IBMX. This pretreatment with IBMX allows cGMP to be quantified without any degradation by PDE. However, platelet cGMP was also quantified without IBMX to allow the effect of AGE on cGMP and associated signalling to be observed.

cGMP concentrations were measured in both resting inactive platelets and ADP activated platelets, using the Biotrak cGMP EIA (GE Healthcare), as described in Chapter 2.6. As reported by Mo *et al* (2004) after aggregation is induced by ADP a transient peak of cGMP should occur approximately 10-20 seconds after the initial point of activation, giving an assumed average peak at 15 seconds. At 15 seconds after activation cGMP should be sufficiently elevated indicative of the response to the respective stimulus. At the end of this period platelets were placed on ice to stop activation. The effect of the vehicle control DMSO in which inhibitors were reconstituted on platelet cGMP was also investigated.

Platelets in a resting state that were pretreated with IBMX exhibited a significant increase in cGMP after being challenged with 5 % (v/v) of AGE to a concentration of 246 ± 24.3 fmol from 156.9 ± 13.09 fmol of the control basal concentration. The vehicle control of diluted DMSO also increased platelet cGMP concentrations to 188.1 ± 3 fmol from 156.9 ± 13.09 fmol of the control concentrations. Platelets treated with ODQ displayed no significant difference in cGMP concentrations to the corresponding vehicle control data. The increase in intraplatelet cGMP induced by AGE was significantly reduced in the presence of ODQ to 165.4 ± 3.13 fmol from 246 ± 24.3 fmol of 5 % (v/v) of AGE alone (Fig.4.11).

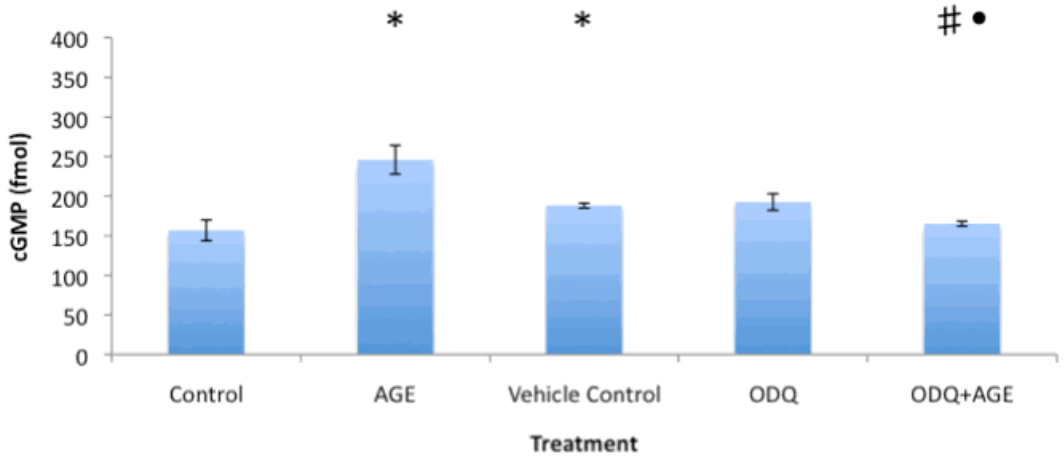


Fig. 4.11:- Resting platelet cGMP concentrations after treatment with 5 % AGE and ODQ. Average intraplatelet cGMP concentrations ± SEM after treatment first with IBMX before ODQ and further challenged with 5 % (v/v) AGE. Vehicle control shows platelets treated with diluted DMSO, which the inhibitor ODQ is dissolved in. Statistically significant data compared to control are indicated with * above respective point with AGE p = 0.028 and the vehicle control p = 0.044. Significant data to ODQ control is indicated with # above data point with p = 0.41. Statistically significant data compared to AGE data is indicated via • above respective point with p = 0.036. All data was calculated using a paired students t test (n=4).

When comparing the controls from Fig. 4.11, and Fig. 4.12 ADP activated platelets with IBMX displayed an increase to 263 ± 16.9 fmol from 156.9 ± 13.09 fmol of platelets in a resting state. As cGMP is an inhibitory molecule it is expected that concentrations in activated platelets should decrease upon activation. However it is currently speculated that cGMP may also be involved in the activation of platelets (Siess 2004).

In contrast to the results observed in the resting platelets, AGE failed to significantly increase cGMP in activated platelets as the concentration of cGMP of 287 ± 8.7 fmol was within the error margins of the control data. The diluted DMSO vehicle control displayed concentrations of 239 ± 8.8 fmol indicating the vehicle did not effect cGMP concentrations, however the presence of the sGC inhibitor ODQ significantly decreased platelet cGMP as expected to 141.3 ± 10.2 fmol. AGE alone failed to increase cGMP in activated platelets, however pretreatment with ODQ caused a decrease in cGMP to 209.4 ± 18 fmol from 287 ± 8.7 fmol of AGE alone. Interestingly the decrease in cGMP with AGE and ODQ did not match the decrease that ODQ alone induced (Fig. 4.12).

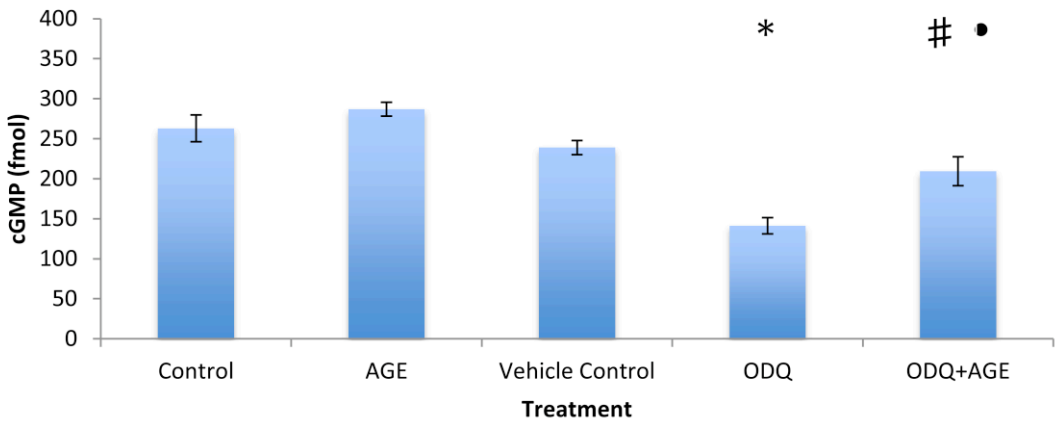


Fig. 4.12:- ADP activated platelet cGMP concentrations after treatment with AGE and ODQ. Average intraplatelet cGMP concentrations ± SEM after treatment first with IBMX before ODQ and further challenged with 5 % (v/v) AGE. Activation was initiated via the addition ADP. Vehicle control shows platelets treated with diluted DMSO, which the inhibitor ODQ is dissolved in. Statistically significant data compared to the vehicle control is indicated with * above respective point p = 0.046. Significant data to ODQ control is indicated with # above data point with p = 0.033. Statistically significant data compared to AGE data is indicated via • above respective point with p = 0.038. All data was calculated via a paired students t test (n=4).

ADP activated platelets with no IBMX pretreatment showed general diminished cGMP concentrations than in the presence of IBMX (Fig 4.13). cGMP concentrations for the control were 96.2 ± 20.2 fmol, with concentrations in AGE treated platelets 117 ± 6.6 fmol and the diluted DMSO vehicle control 103 ± 12 fmol showing no significant increase. Platelets treated with ODQ demonstrably reduced intraplatelet cGMP to 5.7 ± 1.2 fmol from 103 ± 12 fmol, an almost imperceptible level. Platelets challenged with AGE after ODQ, increased cGMP to 51.9 ± 4.4 fmol compared to ODQ alone 5.7 ± 1.2 fmol, however, significantly reduced cGMP in contrast to AGE alone concentrations of 117 ± 6.6 fmol (Fig. 4.13).

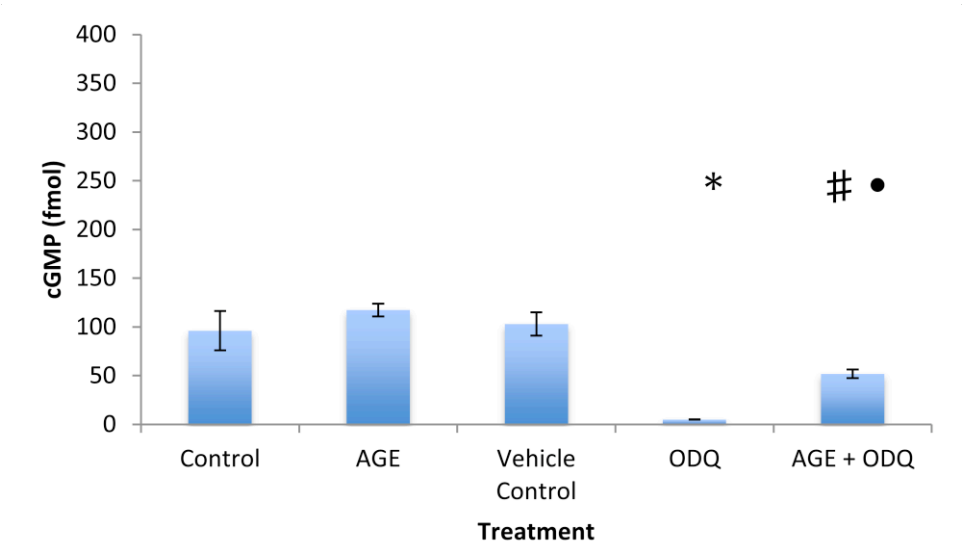


Fig. 4.13:- Intraplatelet cGMP concentration after challenging with 5 % AGE and ODQ, in ADP activated platelets in the absence of IBMX. Average intraplatelet cGMP concentrations ± SEM after treatment with ODQ before further treatment with 5 % (v/v) AGE. Activation was initiated by the addition of ADP. Vehicle control shows platelets treated with diluted DMSO, which the inhibitor ODQ is dissolved in. Statistically significant data compared to the vehicle control is indicated with * above respective point with p = 0.008. Significant data to ODQ control is indicated with # above data point with p = 0.027. Statistically significant data compared to AGE data is indicated via • above respective point with p = 0.03. All data was calculated via a paired students t test (n=4).

Investigations using 5 % (v/v) AGE displayed some conflicting results, therefore a lower concentration of AGE at 1 % (v/v) was also investigated

4.4.2.2 The effect of 1 % AGE and ODQ upon intraplatelet cGMP concentration

At 1 % (v/v) AGE, platelet aggregation is significantly inhibited hence the effect of this dosage of AGE upon intraplatelet cGMP was also investigated. Platelets were challenged with 1 % (v/v) AGE in both resting state and in ADP activated platelets. Isolated platelets were initially pretreated with IBMX to allow the accumulation of the normally transitory molecule.

Concentrations of cGMP in resting platelets were significantly increased after stimulus with 1 % (v/v) AGE to 223 ± 27.2 fmol compared to the resting platelet control

concentration of 156.9 ± 13.09 fmol (Fig. 4.14). The inhibitor ODQ, was diluted in DMSO therefore an appropriate vehicle control was tested using a further dilution of DMSO ≤ 0.5 % (v/v). The total DMSO in this reaction remained ≤ 0.5 % (v/v) even with the additional diluted DMSO found due the presence of IBMX. The addition of further diluted DMSO increased platelet cGMP to 188.1 ± 5.05 fmol relative to the control cGMP measurements of 156.9 ± 13.09 fmol. The addition of ODQ to resting platelets also increased cGMP concentrations to 192.6 ± 10.24 fmol from the control value of 156.9 ± 13.09 fmol. This increase however, was not significant to the vehicle control and can therefore be attributed to the presence of DMSO. The combined effect of ODQ and 1 % (v/v) AGE displayed concentrations of 177.5 ± 1.26 fmol indicating no effect on intraplatelet cGMP concentrations when compared to the ODQ control concentration of 192.6 ± 10.24 fmol. The presence of ODQ did however, significantly decrease cGMP concentrations compared to 1 % (v/v) AGE alone (Fig. 4.14).

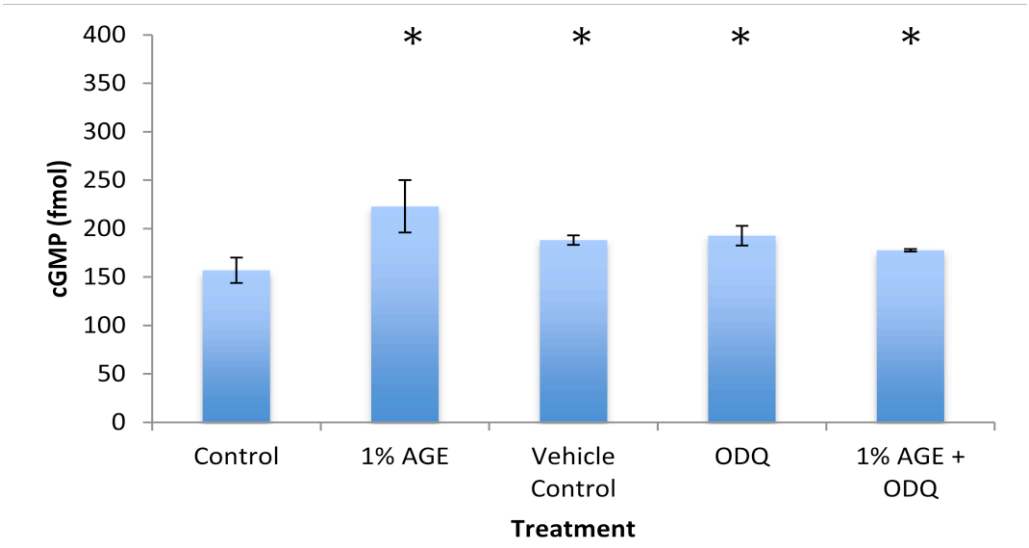


Fig. 4.14:- Resting platelet cGMP concentration after treatment with 1 % AGE and ODQ. Average intraplatelet cGMP concentrations ± SEM after treatment first with IBMX before ODQ were further challenged with 1 % (v/v) AGE. Vehicle control shows platelets treated with diluted DMSO, which the inhibitor ODQ is dissolved in. Statistically significant data compared to control are indicated with * above respective point. Statistically significant data compared to AGE data is indicated via • above respective point. All data has $p \leq 0.04$ and calculated via a paired students t test (n=4).

Concentrations of intraplatelet cGMP were also quantified in ADP activated platelets. Platelets were pretreated with IBMX, before challenged with a respective stimulus. Activation was initiated with ADP and terminated 15 seconds after the addition of ADP to the isolated platelets. ADP activated platelets had cGMP concentrations of 243 ± 16.9 fmol, which displayed an increase in cGMP concentrations in comparison to resting platelets (Fig. 4.15). Platelets challenged with 1 % (v/v) AGE significantly increased basal cGMP concentrations to 273 ± 8.74 fmol from 243 ± 16.9 fmol in ADP activated platelets. The diluted DMSO vehicle control displayed no effect upon activated platelet cGMP concentrations. In the presence of the sGC inhibitor ODQ, cGMP was significantly reduced to 141.3 ± 10.2 fmol in comparison to both the control and vehicle control. Platelets challenged with both 1 % (v/v) AGE and ODQ decreased cGMP to 194 ± 16.4 fmol from 273 ± 8.74 fmol of AGE alone. This reduction in cGMP however is not significant to cGMP concentrations found in the control ADP activated platelets. The presence of ODQ prevents an increase in cGMP induced by AGE, as intraplatelet cGMP concentrations are within error range of the control data (Fig. 4.15).

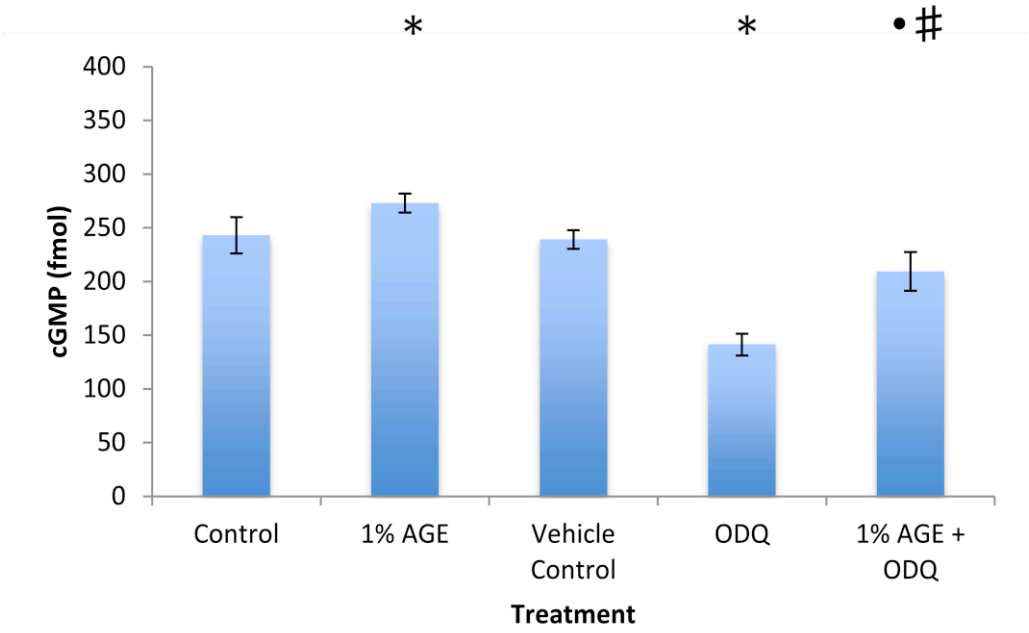


Fig. 4.15:- ADP activated intraplatelet cGMP concentration after treatment with 1 % AGE and ODQ. Average intraplatelet cGMP concentrations ± SEM after treatment first with IBMX before ODQ and further treatment with 1 % (v/v) AGE (v/v). Activation was initiated by the addition of ADP. Vehicle control shows platelets treated with diluted DMSO, which the inhibitor ODQ is dissolved in. Statistically significant data compared to the vehicle control is indicated with * above respective point p = 0.049. Significant data to ODQ control is indicated with # above data point with p = 0.46. Statistically significant data compared to AGE data is indicated via • above respective point with p = 0.04. All data was calculated via a paired students t test to the comparable control (n=4).

4.4.3 The effect of AGE upon platelet cAMP concentration

4.4.3.1 The effect of 5 % AGE and SQ 22536 upon intraplatelet cAMP concentration

AGE can elevate intraplatelet cAMP (Allison 2006) hence this may be one mechanism by which it inhibits platelet aggregation. It is therefore likely that AGE directly stimulates AC as this enzyme is responsible for the synthesis of cAMP. To investigate an inhibitor of AC, SQ 22536 was utilized and cAMP levels were directly quantified in stimulated platelets after treatment with AGE.

To determine the full effect of AGE upon cAMP, the cyclic nucleotide was quantified both in the presence and absence of the PDE inhibitor IBMX. In the presence of IBMX, platelet PDE will be inhibited therefore unable to degrade cAMP. As cAMP is synthesised through the stimulation of AC, this membrane bound enzyme was also inhibited to establish the effect of AGE upon cAMP, therefore platelets were incubated with 100 μM of the AC inhibitor SQ 22536 before being challenged with 5 % (v/v) of AGE. To investigate the effect of AGE upon cAMP thoroughly, the combined effect of AGE and SQ 22536 upon cAMP was quantified in both resting and ADP activated platelets.

In the presence of IBMX the resting intraplatelet basal cAMP concentration was 3951 ± 183 fmol, this was significantly increased to 5858 ± 450 fmol by the presence of 5 % (v/v) of AGE. Resting platelets treated with SQ22536 displayed no significant difference to basal concentrations of cAMP, however the presence of SQ22536 failed to reverse the rise in cAMP induced by 5 % (v/v) of AGE. In the presence of SQ 22536 and 5 % (v/v) AGE intraplatelet cAMP concentrations of 5063 ± 571 fmol are within the error margins of samples treated with only 5 % (v/v) of AGE (Fig. 4.16).

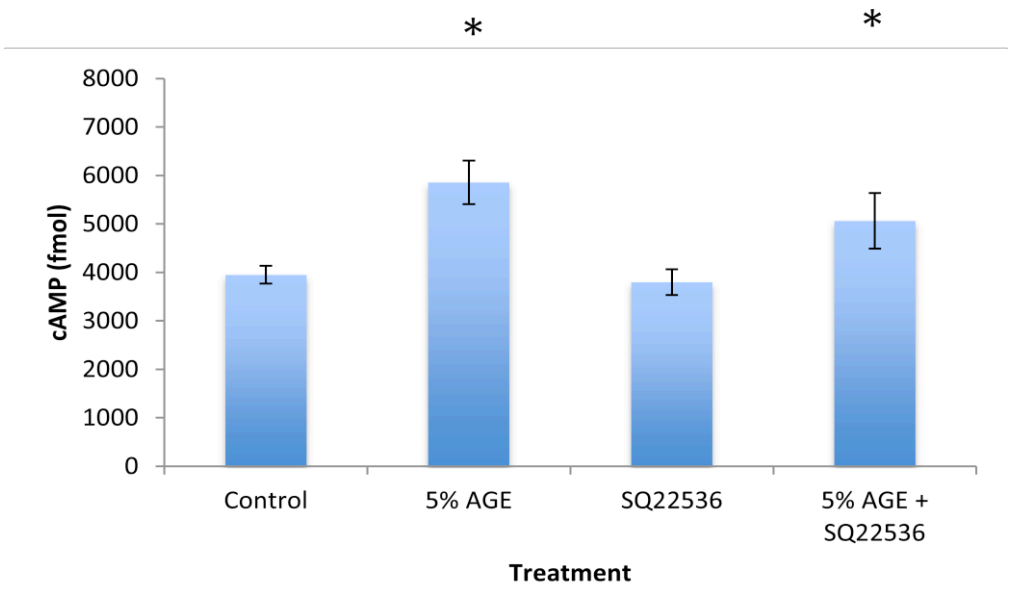


Fig 4.16:- The effect of AC inhibitor SQ 22536 and 5 % AGE on resting platelet cAMP in the presence of IBMX. Average intraplatelet cAMP concentrations ± SEM after treatment first with IBMX, followed by 5 % (v/v) of AGE. Resting platelets are represented by the control. SQ22536 platelets were treated with the AC inhibitor before further treatment with AGE. Statistically significant data compared to control are indicated with * above respective point, with p=0.04 via a paired students t test (n=6).

Concentrations of cAMP in ADP activated platelets were also quantified in the presence of IBMX (Fig. 4.17). Platelets were first treated with IBMX for a time sufficient enough to allow the inhibitor to permeate the cell. Aliquots of platelets were further incubated with either SQ 22536 or ODQ or both before being challenged with 5 % (v/v) AGE. Treated platelets were activated by the addition of 8 µM ADP and the reaction was stopped after 15 seconds, a point at which sufficient cAMP should have accumulated to measure the response provoked by the stimulus, AGE (Mo *et al* 2004). Basal concentrations of cAMP 1205 ± 102 fmol, were significantly increased by 5 % (v/v) AGE to 2078 ± 370 fmol. Incubation with SQ 22536 substantially diminished intraplatelet cAMP concentrations from 1205 ± 102 fmol to 359 ± 51 fmol. The presence of the AC inhibitor failed to fully reverse the increase in cAMP in ADP activated platelets that is induced by 5 % (v/v) AGE with cAMP concentrations of 1759 ± 173 fmol (Fig. 4.17), the same observation was found in resting platelets (Fig. 4.16).

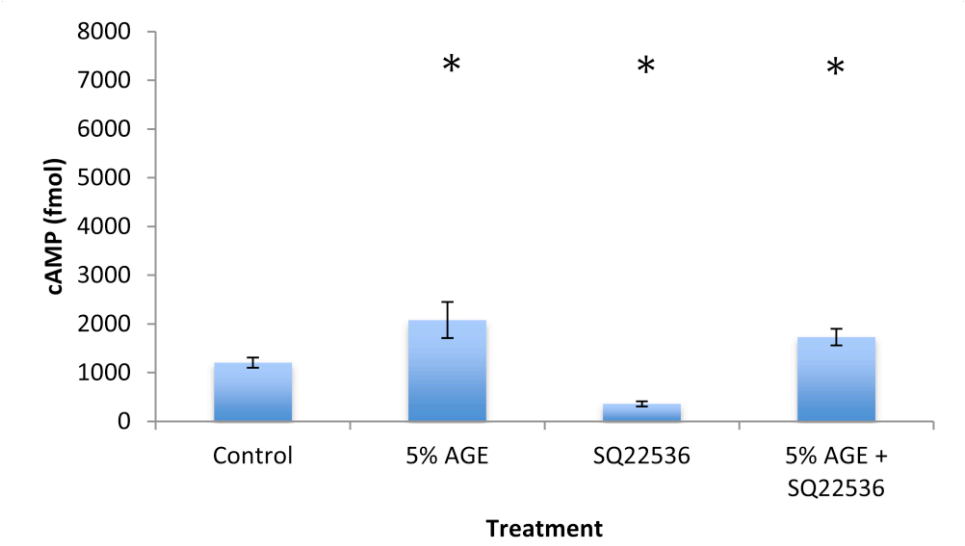


Fig 4.17:- The effect of AC inhibitor SQ 22536 and 5 % AGE on activated platelet cAMP in the presence of IBMX. Average intraplatelet cAMP concentrations ± SEM after treatment first with IBMX, followed by SQ 22536. Platelets were further treated with 5 % (v/v) of AGE and activated by the addition of ADP. Statistically significant data compared to control are indicated with * above respective point, with p 5% AGE p = 0.045, SQ22536 p = 0.03 and 5% AGE + SQ22536 p = 0.037 calculated via a paired students t test (n=6).

Intraplatelet cAMP was also quantified in isolated platelets that were activated by ADP without IBMX pretreatment (Fig. 4.18). Concentrations of cAMP with no IBMX pretreatment show decreased levels at each respective stimulus in comparison to ADP activated platelets with IBMX present. Basal concentrations of cAMP at 860 ± 209 fmol were significantly increased after being challenged with 5 % (v/v) AGE to 1547 ± 132 fmol. The AC inhibitor SQ 22536 decreased cAMP concentrations considerably to 280 ± 36 fmol. This decrease was expected as ADP activation of platelets includes the inhibition of AC therefore prohibiting any further synthesis of cAMP. Platelets treated with both SQ 22536 and 5 % (v/v) AGE, displayed a decrease in cAMP to 647 ± 88 fmol, however despite the cAMP concentrations being significantly higher than SQ 22536 alone, they are comparable to basal concentrations of cAMP (Fig. 4.18).

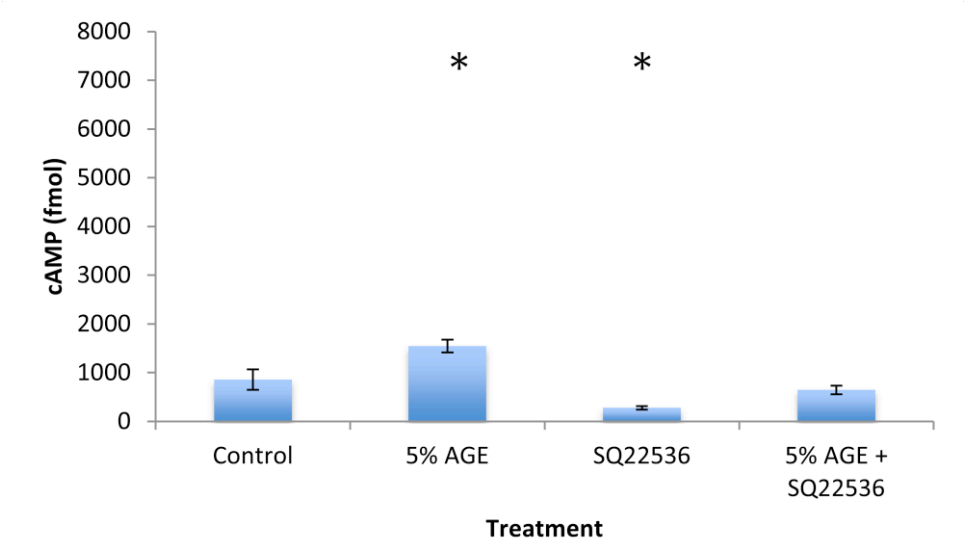


Fig 4.18:- The effect of AC inhibitor SQ 22536 and 5 % AGE on ADP activated platelets cAMP in the absence of IBMX. Average intraplatelet cAMP concentrations ± SEM after treatment with 1 % (v/v) AGE. SQ22536 platelets were treated with the AC inhibitor before being challenged with AGE and activated by the addition of ADP. Statistically significant data compared to control are indicated with * above respective point, with p = 0.038 for 5 % AGE and p = 0.019 for SQ 22536 calculated using a paired students t test (n=6).

4.4.3.2 The effect of 1 % AGE on intraplatelet cAMP concentration

As there were some inconsistent results obtained using 5 % (v/v) AGE and the inhibitor SQ 22536 suggesting that AGE may interfere with the binding of SQ 22536, a lesser concentration of 1 % (v/v) AGE was used to determine the effect upon cAMP.

Intraplatelet cAMP in response to 1 % (v/v) of AGE was quantified both in the presence and absence of IBMX in both resting and ADP activated platelets. Isolated platelets were first treated with IBMX before incubation with SQ 22536 and further challenged with 1 % (v/v) AGE. In platelets activated with ADP activation was stopped after 15 seconds (Mo *et al* 2004) and intraplatelet cAMP quantified using the cAMP BioTrak EIA (GE Healthcare) kit.

In the presence of IBMX resting platelet cAMP was measured at 3951 ± 183 fmol and was increased after treatment with 1 % (v/v) AGE to 4355 ± 316 fmol, however, this rise in cAMP was not significant. In platelet samples treated with the AC inhibitor SQ 22536 cAMP concentrations of 3798 ± 266 fmol displayed no difference to the control

concentrations of 3951 ± 183 fmol. In the presence of SQ 22536 and 1 % (v/v) AGE, cAMP was significantly reduced to 2957 ± 494 fmol from 4355 ± 316 fmol of AGE alone data (Fig. 4.19).

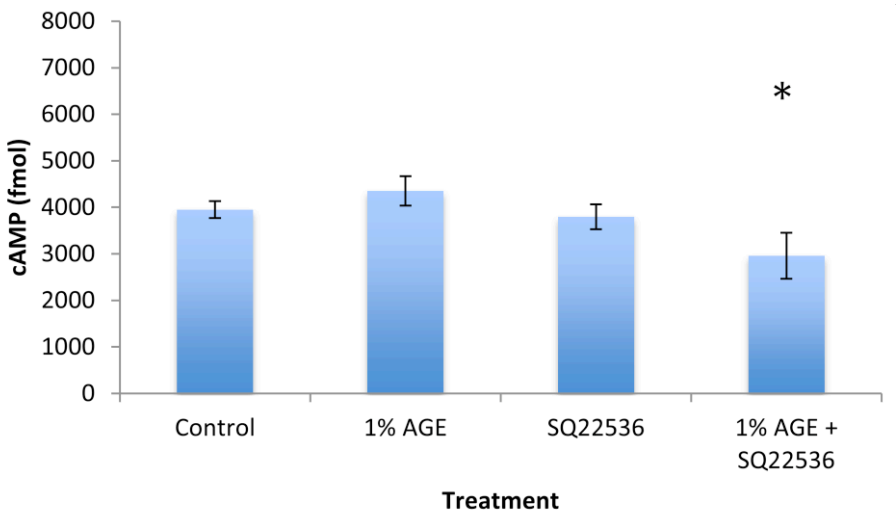


Fig 4.19:- The effect of AC inhibitor SQ 22536 and 1 % AGE on resting platelet cAMP in the presence of IBMX. Average intraplatelet cAMP concentrations ± SEM after treatment first with IBMX, followed by 1 % (v/v) AGE. SQ22536 platelets were treated with the AC inhibitor before further treatment with AGE. Statistically significant data compared to 1 % (v/v) of AGE are indicated with * above respective point, with p = 0.027 via a paired students t test (n=6).

Concentrations of cAMP in ADP activated platelets (Fig. 4.20) in the presence of IBMX were increased to 1465 ± 96 fmol from 1205 ± 102 fmol after being challenged with 1 % (v/v) of AGE. As expected, SQ 22536 significantly decreased cAMP to 359 ± 51 fmol when compared to the ADP activated control. The inhibitor SQ 22536 prevented an increase in cAMP induced by 1 % (v/v) AGE with concentrations of 450 ± 162 fmol, suggesting that this lower concentration of AGE does not affect the binding of the inhibitor (Fig. 4.20).

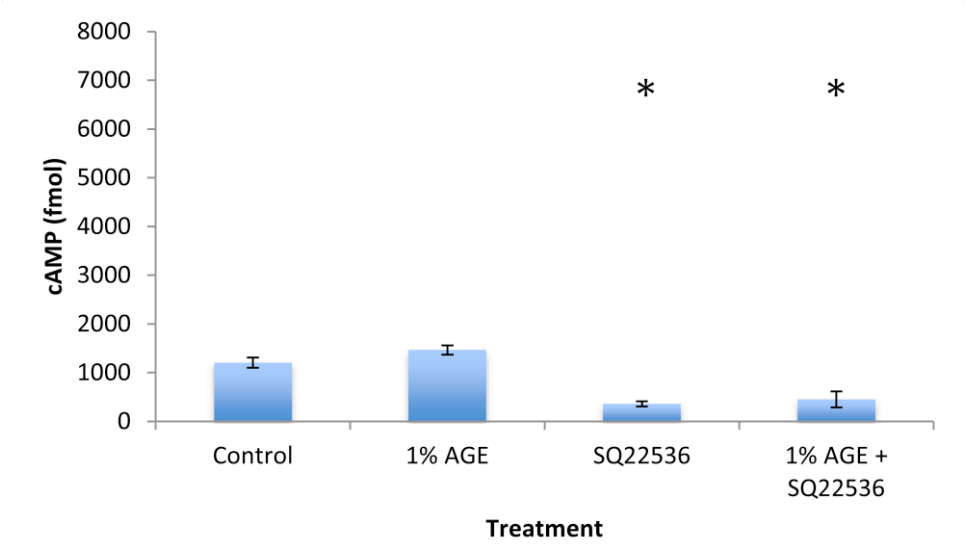


Fig 4.20:- The effect of AC inhibitor SQ 22536 and 1 % AGE on activated platelet cAMP in the presence of IBMX. Average intraplatelet cAMP concentrations ± SEM after treatment first with IBMX, followed by SQ 22536. Platelets were further challenged with 1 % (v/v) AGE before activation was initiated by ADP. Statistically significant data compared to control are indicated with * above respective point, with p = 0.02 via a paired students t test (n=6).

In the absence of the PDE inhibitor IBMX ADP activated platelets showed overall diminished concentrations of cAMP when compared to the results obtained with IBMX. Activated platelet cAMP concentrations of 860 ± 209 fmol were increased by 1 % (v/v) AGE however this increase was not significant. The AC inhibitor SQ 22536, as expected, significantly decreased intraplatelet cAMP to 280 ± 36 fmol from 860 ± 209 fmol of the control. In the presence of SQ 22536, AGE significantly increased cAMP concentrations to 504 ± 28 fmol when compared to the inhibitor SQ 22536 alone. This increase, however, did not surmount to the control or AGE alone concentrations, suggesting that the AC inhibitor is preventing the full stimulatory effects of AGE upon platelet cAMP (Fig. 4.21).

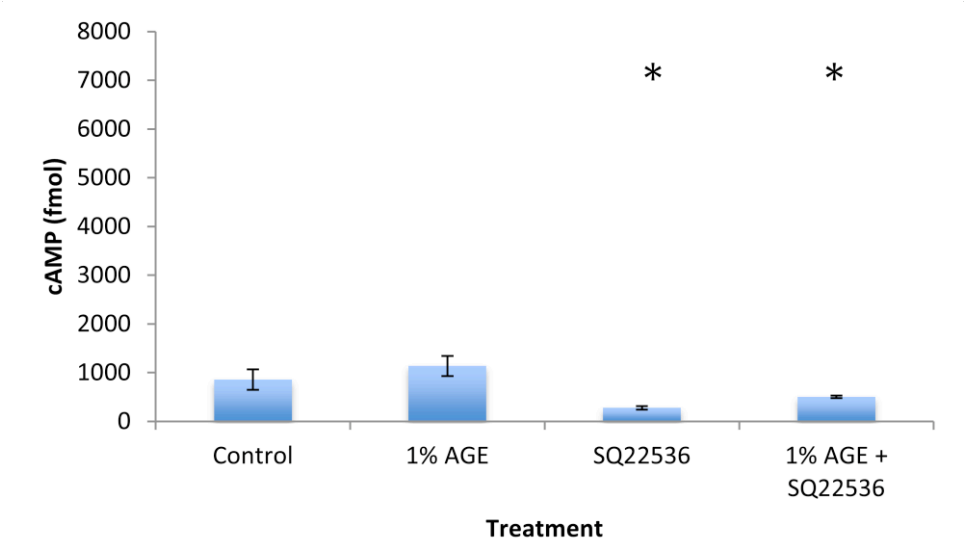


Fig 4.21:- The effect of AC inhibitor SQ 22536 and 1 % AGE on ADP activated platelets cAMP in the absence of IBMX. Average intraplatelet cAMP concentrations ± SEM after treatment with 1 % (v/v) AGE. SQ22536 platelets were treated the AC inhibitor before further treatment with AGE and were activated by the addition of ADP. Statistically significant data compared to control are indicated with * above respective point, with p = 0.021 via a paired students t test (n=6).

4.4.4 The effect of AGE and the inhibitors ODQ and SQ 22536 upon intraplatelet cyclic nucleotides

Earlier experiments had suggested that concentrations of AGE at 5 % (v/v) or higher, may have been influencing the binding of the inhibitors or removing the inhibitors from their respective active sites (Fig. 4.6, 4.7, 4.11, 4.16). Due to this potential technical experimentation problem, concentrations of 1 % (v/v) AGE were only utilised in further investigations.

Using the inhibitors ODQ and SQ 22536, the full extent of the effect of AGE upon platelet cyclic nucleotides can be established. If AGE directly acts upon cGMP and cAMP, inhibiting both sGC and AC should remove the enhancing effects of AGE upon each nucleotide.

Isolated platelets were first incubated with IBMX before further incubation with both the inhibitors ODQ and SQ22536. Cells were then challenged with 1 % (v/v) of AGE and the effect on both intraplatelet cGMP and cAMP concentrations were quantified.

4.4.4.1 Platelet cGMP in response to AGE whilst inhibiting sGC and AC with ODQ and SQ 22536

Platelet cGMP was quantified in response to AGE whilst in the presence of both inhibitors ODQ and SQ 22536. In resting platelets treated with IBMX cGMP concentrations of 156 ± 13 fmol were significantly increased after being challenged with 1 % (v/v) AGE to 222 ± 24 fmol. The presence of both of the inhibitors ODQ and SQ 22536 had no significant effect upon platelet cGMP despite the partial decrease in cGMP concentrations to 129.5 ± 11 fmol when compared against the control data. AGE at 1 % (v/v) with both ODQ and SQ 22536 significantly decreased resting platelet cGMP concentrations to 115.7 ± 19 fmol when compared control and AGE alone data of 222 ± 24 fmol. As there was no difference between ODQ plus SQ 22536 to 1 % (v/v) AGE plus ODQ and SQ 22536, this suggests that the effect of AGE upon platelet cGMP is being prevented by the presence of both inhibitors (Fig. 4.22).

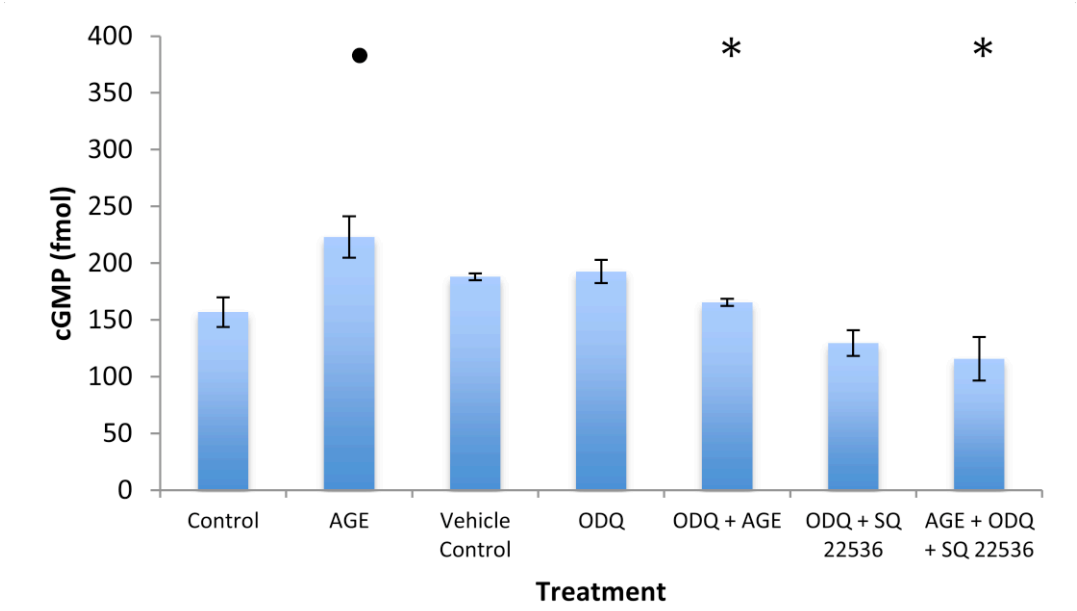


Fig. 4.22:- cGMP concentrations in resting platelets with IBMX in response to AGE, SQ 22536 and ODQ. Average intraplatelet cGMP concentrations ± SEM after treatment first with IBMX followed by 1 % (v/v) AGE. ODQ and SQ22536 platelets were treated with either inhibitor before further challenging with AGE. Statistically significant data compared between treatments via a paired students t-test and are indicated with • above respective point showing to control data with p = 0.03 and * to AGE alone with p = 0.04 (n=6).

In ADP activated platelets (Fig. 4.23) to correspond with other experiments, activation was stopped 15 seconds after the addition of ADP. Control cGMP concentrations in activated platelets with IBMX pretreatment were 263 ± 17 fmol and remained unchanged after treatment with SQ 22536. The presence of the inhibitor ODQ with AGE decreased cGMP concentrations to 210 ± 18 fmol, whereas the AC inhibitor SQ 22536 decreased cGMP to 190 ± 9 fmol. The presence of both inhibitors SQ 22536, ODQ and AGE, also significantly decreased control cGMP concentrations to 162 ± 3 fmol from 263 ± 17 fmol (Fig. 4.23).

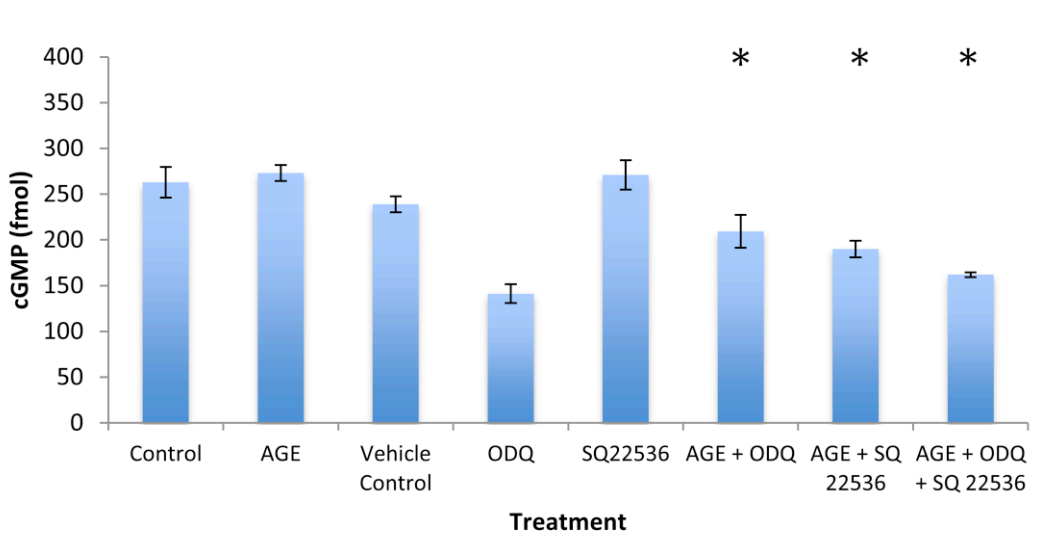


Fig. 4.23:- The effect of AGE, ODQ and SQ22536 on intraplatelet cGMP in activated platelets treated with IBMX. Average intraplatelet cGMP concentrations ± SEM after treatment first with IBMX before incubation with ODQ and SQ22536. Platelets were then challenged with 1 % (v/v) AGE and activated by the addition of ADP. Statistically significant data is indicated with * above respective point to AGE alone data p = 0.04 (n=6).

Activated platelets with no IBMX present (Fig. 4.24), displayed diminished concentrations of cGMP when compared to samples in the presence of the PDE inhibitor. This was expected, as the PDE are active and will act to degrade cGMP, converting the nucleotide into its inactive metabolite. Basal cGMP concentrations of 96 ± 20 fmol were not significantly increased by treatment with 1 % (v/v) AGE or the diluted DMSO vehicle control with concentrations of 117 ± 7 fmol and 103 ± 12 fmol respectively. ODQ substantially decreased basal cGMP concentrations to 5 ± 1 fmol, as did the AC inhibitor SQ 22536 to 17 ± 5 fmol.

The presence of either cyclase inhibitor in the presence of AGE decreased cGMP to 52 ± 4 fmol with ODQ and 17 ± 3 fmol with SQ 22536, when compared to AGE alone The presence of both inhibitors with AGE significantly decreased intraplatelet cGMP to 21 ± 3 fmol when compared to cGMP concentrations of 117 ± 7 fmol for AGE alone. Interestingly the presence of SQ 22536 with AGE had the most effect upon cGMP concentrations, decreasing cGMP more than ODQ plus AGE, suggesting that cAMP may have a more important role (Fig. 4.24).

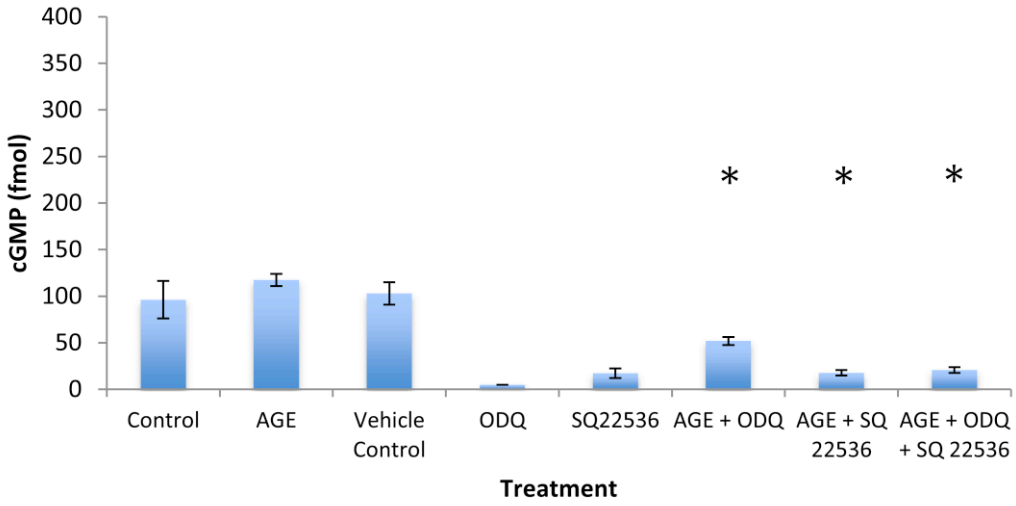


Fig. 4.24:- The effect of AGE, ODQ and SQ22536 on intraplatelet cGMP in activated platelets in the absence of IBMX. Average intraplatelet cGMP concentrations ± SEM after treatment first with ODQ and SQ22536 before further treatment with 1 % (v/v) AGE. Activation was initiated by the addition of ADP. Statistically significant data is indicated with * above respective point to AGE alone data p = 0.025 (n=6).

4.4.4.2 Platelet cAMP in response to AGE whilst inhibiting sGC and AC with ODQ and SQ 22536

Intraplatelet cAMP was quantified in resting platelets in the presence of IBMX. Basal concentrations of cAMP indicated by the control were 3951 ± 183 fmol, this was increased to 4355 ± 316 fmol in platelets treated with 1 % (v/v) AGE, this elevation of cAMP however, was not significant. Incubation with SQ 22536 did not affect platelet cAMP with concentrations of 3798 ± 266 fmol. AGE in the presence of the inhibitor SQ 225536 displayed cAMP concentrations of 2957 ± 494 fmol, which is in range of both the control and AGE alone data.

Diluted DMSO (≤ 0.5 %, v/v) was utilized as a vehicle control and did not affect cAMP concentrations. The combination of SQ 22536 and ODQ acted to significantly decrease control cAMP concentrations from 3951 ± 183 fmol to 3407 ± 257 fmol. The presence of both inhibitors also prevented an increase in cAMP by AGE decreasing cAMP concentrations to 3090 ± 147 fmol (Fig. 4.25).

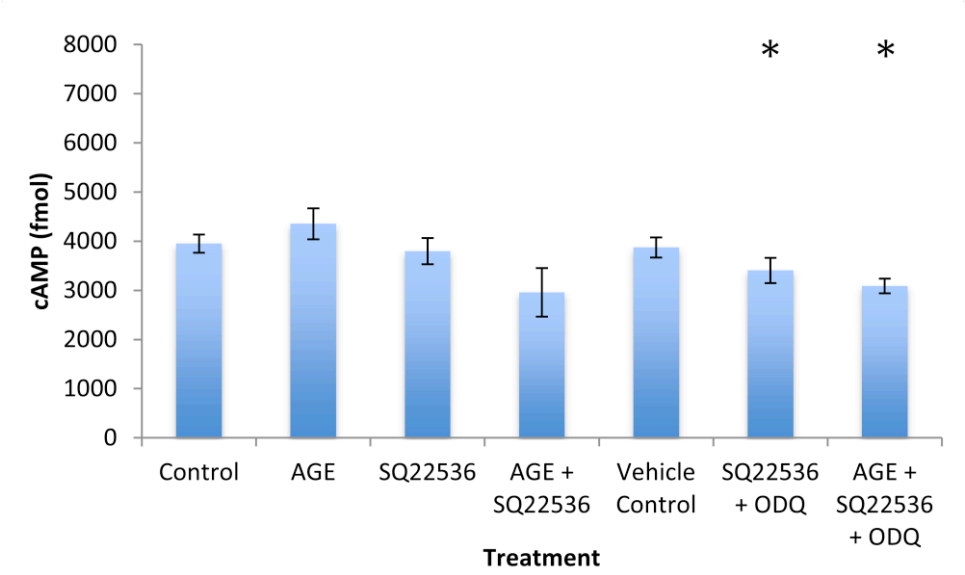


Fig 4.25:- cAMP concentrations in resting platelets in response to AGE, SQ 22536 and ODQ in the presence of IBMX. Average intraplatelet cAMP concentrations ± SEM after treatment first with IBMX then with AGE 1% (v/v). ODQ and SQ22536 platelets were treated with either inhibitor before further treatment with AGE. Statistically significant data compared between treatments via a paired students t-test and are indicated with * to AGE, p = 0.048 (n=6).

Platelets activated with ADP and in the presence of IBMX (Fig. 4.26), displayed decreased control concentrations of cAMP of 1205 ± 102 fmol, when compared to platelets in a resting state with concentrations of cAMP 3951 ± 183 fmol. This reduction is expected and can be attributed to the mechanism of activation by ADP, which inhibits AC. Samples challenged with 1 % (v/v) of AGE showed a significant increase in cAMP to 1465 ± 96 fmol compared to the activated control concentration of 1205 ± 102 fmol.

SQ 22536 considerably decreased cAMP to 359 ± 51 fmol. The presence of this inhibitor also prevented AGE from inducing any effect upon platelet cAMP with cAMP concentrations of 450 ± 162 fmol demonstrably less than AGE alone. The diluted DMSO vehicle control displayed no difference in intraplatelet cAMP and neither did the presence of both SQ 22536 and ODQ with cAMP concentrations 1136 ± 162 fmol and 1604 ± 291 fmol respectively when compared to control data value of 1205 ± 102 fmol. Compared to the control and AGE alone samples, treatment with AGE, SQ 222536 and ODQ significantly lowered cAMP concentrations to 683 ± 106 fmol from 1205 ± 102 fmol or 1465 ± 96 fmol for the control and AGE respectively (Fig. 4.26).

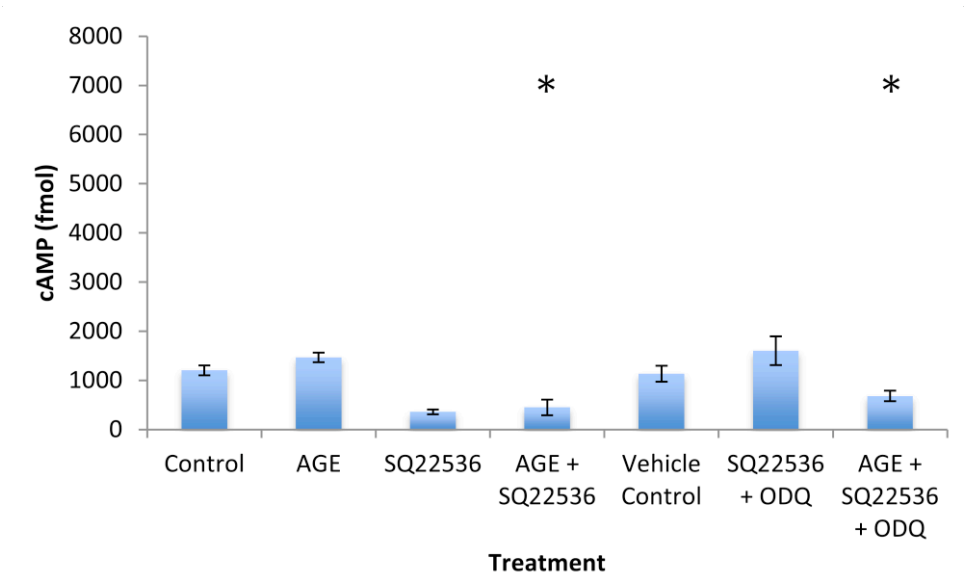


Fig 4.26:- The effect of AGE, ODQ and SQ22536 on intraplatelet cAMP in activated platelets treated with IBMX. Average intraplatelet cAMP concentrations ± SEM after treatment first with IBMX before further incubation with ODQ and SQ22536. Platelets were then challenged with 1 % (v/v) of AGE before ADP initiated activation. Statistically significant data is indicated with * above respective point to AGE alone data with p = 0.02 for AGE + SQ22536 and p = 0.04 for AGE + SQ22536 + ODQ (n=6).

Platelets activated by ADP without IBMX (Fig. 4.27), exhibit decreased cAMP concentrations than that observed in activated platelets with IBMX present. This decrease is expected, due to the degradation of cAMP by the active and uninhibited PDE. Control cAMP concentrations of 860 ± 209 fmol were not significantly increased by 1 % (v/v) of AGE as the cAMP concentrations after treatment with AGE of 1137 ± 207 fmol are within error margins. SQ 22536 considerably decreased intraplatelet cAMP to 280 ± 36 fmol from 860 ± 209 fmol of the control however, the presence of SQ 22536 fails to achieve similar concentrations in the presence of 1 % AGE. SQ 22536 plus AGE cAMP concentrations were 504 ± 28 fmol, suggesting AGE is still able to affect cAMP despite AC being inhibited. The diluted DMSO vehicle and the inhibitors SQ 22536 plus ODQ displayed no difference to the control with cAMP concentrations of 981 ± 147 fmol and 706 ± 164 fmol respectively. The sGC inhibitor ODQ alone did not affect intraplatelet cAMP concentrations (data not shown). Inhibiting both sGC and AC prevented any significant increase in cAMP by 1 % (v/v) AGE, as the cAMP concentration of 408 ± 131 fmol is within range of SQ 22536 alone (Fig. 4.27).

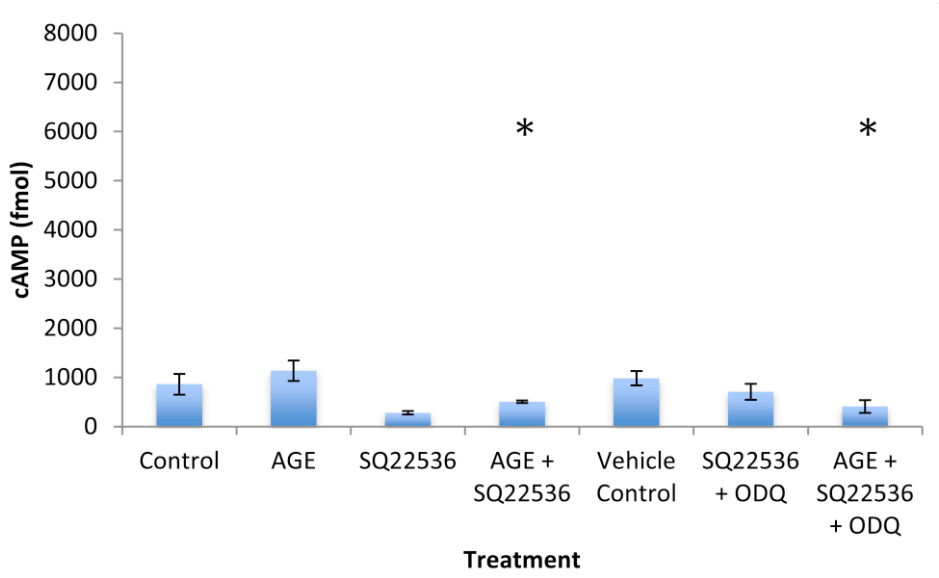


Fig 4.27:- The effect of AGE, ODQ and SQ22536 on intraplatelet cAMP in activated platelets in the absence of IBMX. Average intraplatelet cAMP concentrations ± SEM after treatment first ODQ and SQ22536 before further treatment with 1 % (v/v) AGE. Platelets were activated by the addition of ADP. Statistically significant data is indicated with * above respective point to AGE alone data p = 0.03 (n=6).

4.4.5 The disaggregatory effects of AGE on platelets

Increases in cAMP and cGMP are potent inhibitors of platelet aggregation. The addition of stimulators of either cyclic nucleotide has also indicated the ability to disperse agonist induced platelet aggregates into single platelets. The stimulator of cAMP, PGE_1 displayed an increase in disaggregation of platelets when compared to the cGMP stimulator Sin-1 (Fig. 3.23). As AGE can influence both intraplatelet cAMP and cGMP, using aggregometry, the disaggregation effects of AGE were also investigated.

ADP initiated platelet aggregation and the reaction was allowed to reach maximal aggregation with total percentage aggregation ≥ 65%. AGE was then added at different concentrations to the pre-aggregated PRP and left to run for a total time of 8 min (Fig. 4.28). Disaggregation was calculated against control data as previously stated (Fig. 2.1), as described by Naimushin and Mazurov 2003.

The control does not show any disaggregation and therefore has a relative value of zero. At 1 % (v/v) of AGE the disaggregation achieved 37 % ± 4.5 % and is proportionate to the inhibition of aggregation achieved when platelets are pretreated with AGE (Fig.4.1). Increasing the concentration to 5 % (v/v) of AGE failed to further increase the disaggregation of platelets with a disaggregation percentage of 27 % ± 3.8 % when compared to 1 % (v/v) AGE. However this does not suggest that maximal disaggregation has occurred, as at 10 % (v/v) of AGE, disaggregation was further increased to 67 % ± 2.6 %. Despite this increase at 10 % (v/v) of AGE, 100 % meaning the full dispersal of platelet aggregates into single platelets has occurred was not observed (Fig.4.28). This data does however indicate that AGE, even at low concentrations, can reverse ADP induced platelet aggregates into single platelets.

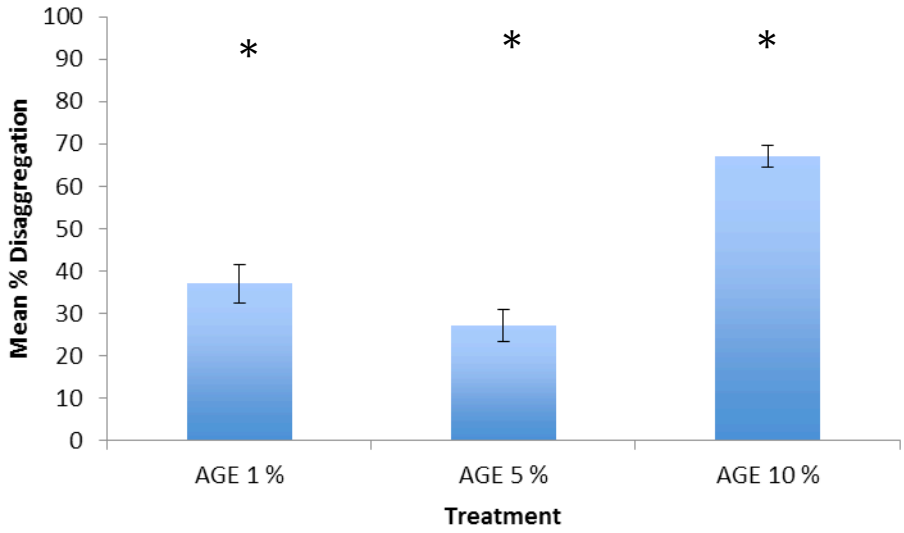


Fig.4.28a:- The diaggregatory effects of AGE on ADP induced platelet aggregation *in vitro*. Aggregation was initiated in PRP via the addition of ADP and once platelets had achieved ≥ 65% aggregation, concentrations of AGE from 1 – 10 % (v/v) were added to PRP. Disaggregation values presented are calculated to the control data value of 0 % disaggregation (not shown). Statistical analysis was performed using an oneway ANOVA with significance indicated by * with $p \leq 0.03$ (n=6).

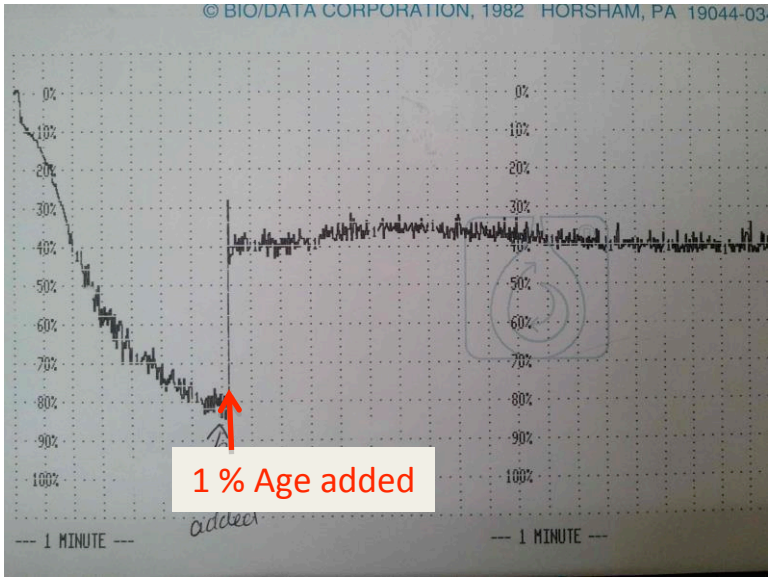


Fig.4.28b:- Representative trace of aggregation indicating the diaggregatory effects of 10 % AGE on ADP induced platelet aggregation *in vitro*. Aggregation was initiated in PRP via the addition of ADP. At maximal aggregation AGE at 1 % (v/v) was added to the aggregated PRP as indicated.

4.4.6 The effect of AGE upon NO metabolites

The addition of Sin-1 to platelets inhibited platelet aggregation (Fig. 3.4) caused the disaggregation of platelets (Fig.3.23), yet failed to significantly increase the nitrosylation of tyrosine residues and nitrite concentrations (Fig. 3.25). As AGE can also influence intraplatelet cGMP, the effect of AGE upon NO and associated metabolites, was of high interest.

4.4.6.1 Measurement of nitrite using Griess assay

A metabolite of NO *in vitro* is nitrite and can be quantified using the Griess assay, which is a simple but crude measurement of nitrite with a detection limit of 3µM.

The effect of AGE upon platelet NO was of interest as the NO donor Sin-1 and AGE causes increased inhibition in platelet aggregation (Fig. 4.3) as well as AGE increasing intraplatelet cGMP (Fig. 4.16). AGE, however has also previously shown an ability to

decrease the presence of free radicals (Dillon *et al* 2003, Morihara *et al* 2011), it was therefore of interest to establish the effect of AGE upon platelet NO. As nitrite is a metabolite of NO, measuring nitrite provides a method of observing NO activity *in vitro*.

Isolated platelets were treated with PBS for the negative control and Sin-1 provided a positive control, as treatment with Sin-1 should invoke an increase in nitrite concentration through the spontaneous production of NO (Cardoso *et al* 2010). Treated platelets were lysed, with the resulting platelet lysate and media kept separate and each analysed individually for nitrite concentrations using the Griess assay. Sin-1 when added to platelets can produce NO, therefore some of this NO may cross the platelet membrane however, the majority will remain in media along with AGE. If AGE can scavenge NO it is expected that NO left in the media produced by Sin-1 in the presence of AGE will be decreased, when compared to platelets treated with only Sin-1.

Platelets were incubated with either Sin-1 or Sin-1 plus 10% (v/v) AGE (final concentration) before being subjected to centrifugation. The supernatant was removed and the platelets were lysed. The concentration of nitrite solely in the pelleted platelet lysate was quantified (Fig. 4.29), as well as the combined media and the lysate samples (Fig. 4.30). These experiments do not measure the amount of nitrite in whole platelets. They provide a crude estimation of nitrite in the medium the platelets are dissolved and in the platelet lysate. This methodology will also provide an indication whether AGE can reduce the amount of nitrite generated by Sin-1. The lysate and supernatant should give a higher reading of nitrite due to the remaining NO from Sin-1 in the supernatant. The lysate itself will only represent what NO has been able to cross the platelet membrane. This experiment solely measured nitrite and did not utilize a further reduction step to measure nitrate hence only one metabolite of NO was measured (Fig. 4.29, Fig 4.30).

For analytical purposes only, a colourless extract of AGE was prepared using diethyl ether. This was to help further eliminate the possibility that the dark colour of AGE may interfere with the assay. The diethyl extract of AGE (DEA) does not contain the same active compounds as the original AGE solution mostly containing non-polar compounds and was used for analytical purposes only.

Measurement of nitrite in platelet lysate samples proved to be inaccurate as the concentrations of nitrite was shown to be less than the sensitivity limit of 3 μM of the

assay. These results therefore cannot be considered to be accurate enough to be a true representation of nitrite concentrations as they are below the detection limits of the assay therefore no statistical analysis was carried out on this data (Fig. 4.29).

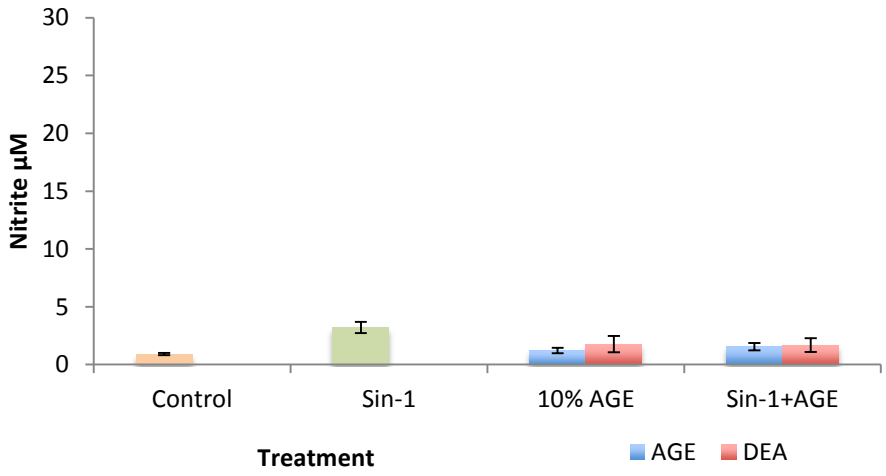


Fig. 4.29:- Total nitrite concentration ± SEM in platelet lysates using 10 % AGE and a diethyl extract of AGE (DEA). Untreated platelets (control, orange) compared to positive control Sin-1 (green), platelets treated 10 % (v/v) AGE or 10 % (v/v) DEA (10 % AGE) or with Sin-1 and 10 % (v/v) of AGE or DEA (Sin-1 + AGE) (final concentrations). All platelets were treated before being lysed. Statistical significance was not calculated due to small sample number and the results being below the detection limit of the assay (n=3).

When quantifying both the media and platelet lysate, as expected there is a significant increase in nitrite concentration in the presence of Sin-1 to 20.6 ± 3.5 µM, from the control value of 5 ± 0.9 µM. AGE at 10% (v/v) alone does not show any interference in the assay, as it displays a similar concentration of nitrite to the control with concentrations of 5.2 ± 0.1 µM. As the Griess assay is colourmetric, several controls were investigated to ensure the dark colour of AGE did not interfere with the assay. The combination of AGE and Sin-1 shows a statistically significant decrease in nitrite concentrations to 7.3 ± 0.4 µM compared to the concentration that Sin-1 alone induced (Fig.4.30).

The presence of 10 % (v/v) of the DEA extract caused a decrease in nitrite to 3.27 ± 0.1 µM, compared to the control concentration of 5 ± 0.9 µM. DEA also decreased nitrite after treatment with Sin-1 to 3.2 ± 0.1 µM. These concentrations, however, are very close

to the detection limits of the Griess assay and as such may not provide an accurate depiction of nitrite within the sample; therefore these results were not included in any statistical analysis (Fig. 4.30).

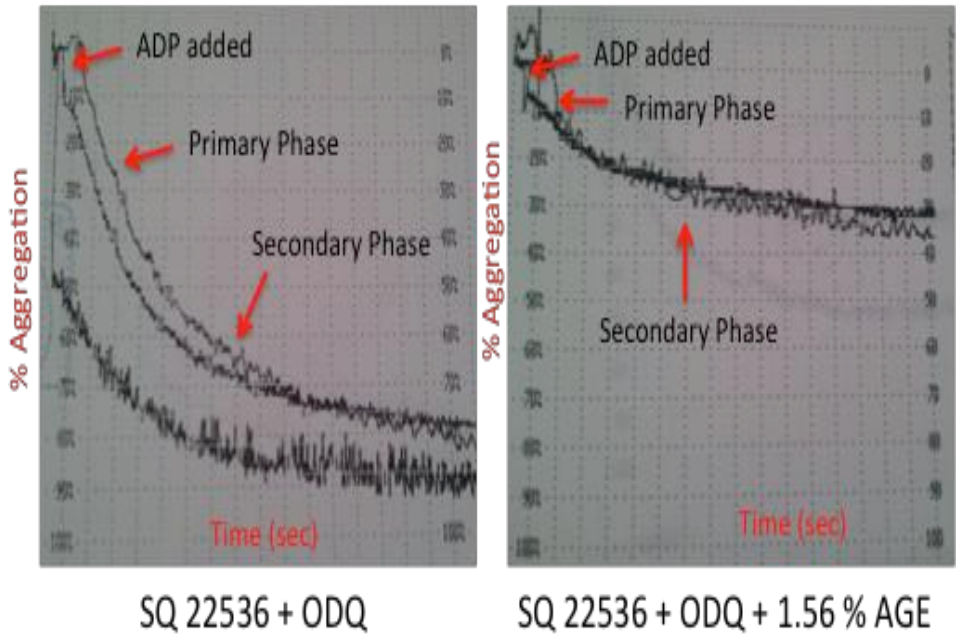


Fig.4.30:- Total nitrite concentration ± SEM in lysed platelets and their media supernatant using 10 % AGE and a diethyl extract of AGE (DEA). Untreated platelets (control, orange), compared to positive control Sin-1 (green), platelets treated with 10 % (v/v) AGE or 10 % (v/v) DEA (10 % AGE), Sin-1 and 10 % (v/v) of AGE or DEA (Sin-1 + AGE) (final concentrations). All platelets were treated before being lysed. Statistical significance was calculated between Sin-1 alone data and Sin-1 + AGE via students t test with p = 0.019 indicated by * above respective point. ◆ indicates significance compared to control data with p = 0.009 for Sin-1 and p = 0.038 for Sin-1+AGE data (n=6).

Concentrations of AGE ranging from 0.5 – 10 % (v/v) were investigated upon nitrite contained in both the media and platelet lysate. The presence of AGE at any concentration did not affect nitrite concentration within the samples compared to the control (Fig.4.31).

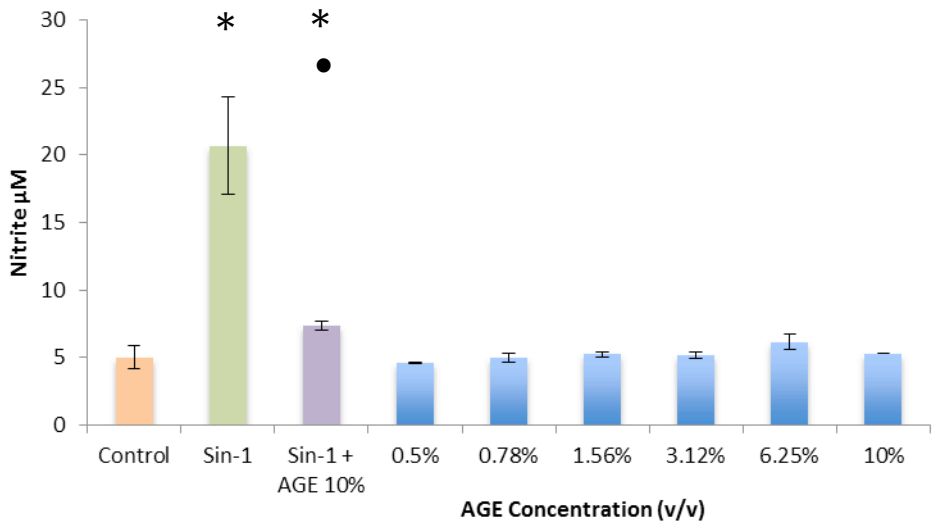


Fig. 4.31:- Total nitrite concentration ± SEM in the media and pellet of lysed platelets using differing concentrations of AGE. Control platelets (orange), compared to positive control Sin-1 (green), platelets treated with Sin-1 plus 10 % (v/v) AGE (purple) and concentrations of AGE ranging between 0.5 – 10 % (v/v). All platelets were treated before being lysed with the presented values representing nitrite found in both media and platelet pellet. Statistical significance is calculated against the control via students t test with p = 0.0.009 for Sin-1 and p = 0.038 for Sin-1+AGE indicated by * above respective point. • indicates significant data to Sin-1 control with p = 0.019 (n=6).

4.4.6.2 The interference of AGE in the fluorescent detection of NO in vitro

To increase the detection sensitivity of nitrite within a platelet sample, a fluorescent NO assay kit was obtained (Calbiochem, UK). This would allow the detection of nitrite to a minimum of 30 nM per well, which would equate to approximate concentration of 0.2 µM within the sample, substantially increasing the sensitivity beyond the 3 µM limits of the Griess assay.

The assay can measure both nitrate and nitrite and is based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The addition of 2,3-diaminonapthalene (DAN) and NaOH converts nitrite to a fluorescent compound 1(H)-naphthotriazole. Fluorescence measurements of this compound using a fluorometer will therefore determine the nitrite (NO_2^-) concentration in the sample.

AGE itself possesses some auto-fluorescent properties, as was previously identified (Allison, PhD Thesis, 2007). To establish whether these fluorescent properties or the dark colour of AGE would interfere with this particular assay, both AGE and the colourless diethyl extract of AGE were added to a 0.3 µM standard, which equated to a concentration of 15.6 pmol per well. This initial experiment utilized only nitrite standards and no platelets were present.

The nitrite standard alone was correctly measured at 15.6 pmol, however in the presence of both AGE and the diethyl extract highly unusual and inaccurate negative results were observed (Fig. 4.32). These technical errors could not be overcome, as samples containing AGE continued to produce unreliable results, therefore no further experiments or analysis were undertaken (Fig. 4.32).

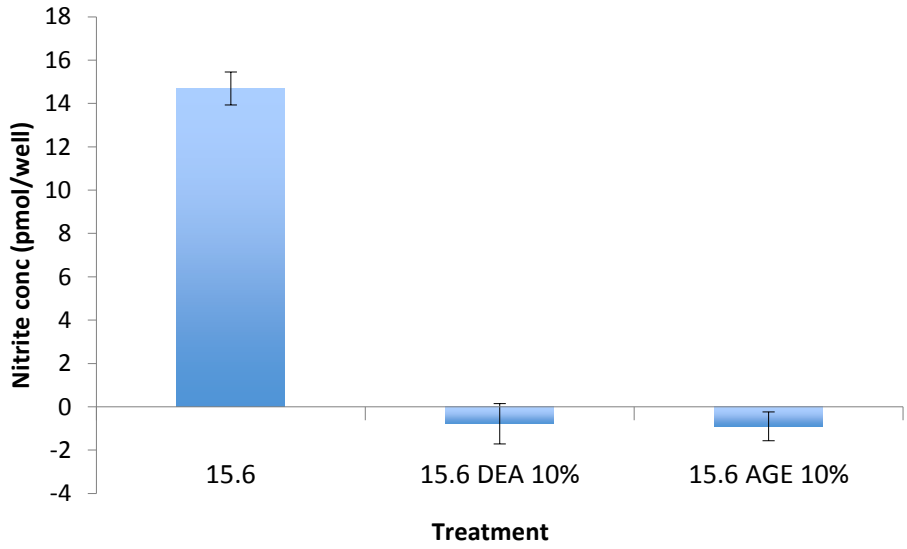


Fig. 4.32:- Nitrite concentration using a fluorescent NO kit. A standard of 0.3 µM equaling to 15.6 pmol / well was used ± SEM. 10 % (v/v) AGE and 10 % (v/v) DEA (final concentrations) were added to the same standard. No platelets were present in this experiment. No statistical analysis was performed on this data (n=3).

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4.4.6.3 Platelet nitrotyrosine expression after treatment with AGE

Quantifying the presence of nitrated proteins can serve as another marker for the activity of NO *in vivo* and can be investigated using a non-colourmetric method, thus eliminating the potential interference of AGE with the experiment. The generation of peroxynitrite by Sin-1 should induce the nitration of proteins and therefore increase the expression of nitrotyrosine (Cardoso *et al* 2010). Nitrotyrosine can be measured in numerous ways, with the most common using specific antibodies to detect any difference in the expression of the protein. Quantifying the expression of nitrotyrosine in the presence and absence of both AGE and Sin-1 will help to elucidate the effect if any, that AGE has upon platelet NO and corresponding NO metabolites.

The technique was first tested upon nitrated human serum albumin (HSA) to ensure correct methodology and to establish suitable concentrations of both primary and secondary antibodies (Fig. 3. 25). Treatment of HSA and platelets with Sin-1 increased the expression of nitrotyrosine due to the peroxynitrite generated from the NO chemical mimetic.

As the methodology was proved to be working correctly, the experiments were repeated using platelet samples. Isolated platelets were treated with PBS (negative control) or Sin-1. Separate samples of platelets were also treated with 10 % (v/v) AGE or a combination of Sin-1 and 10 % (v/v) AGE (final concentration). Crude lysates were made of the appropriate treated platelets and protein concentration per sample was determined using a Bradford assay. Proteins were separated using SDS PAGE with 40 µg of protein loaded per sample lane. Two gels were run simultaneously, one for commassie blue staining and one for transfer to membrane for antibody probing.

One of the 15 % (w/v) polyacrylamide gels with the corresponding proteins loaded was stained with coomassie brilliant blue. The expected weight of nitrotyrosine was 67 kDa, as indicated with the nitrated HSA standard. Proteins with corresponding molecular weight as nitrated HSA, displayed no significant difference in expression across all treatments in the stained SDS polyacrylcamide gel (Fig. 4.33a).

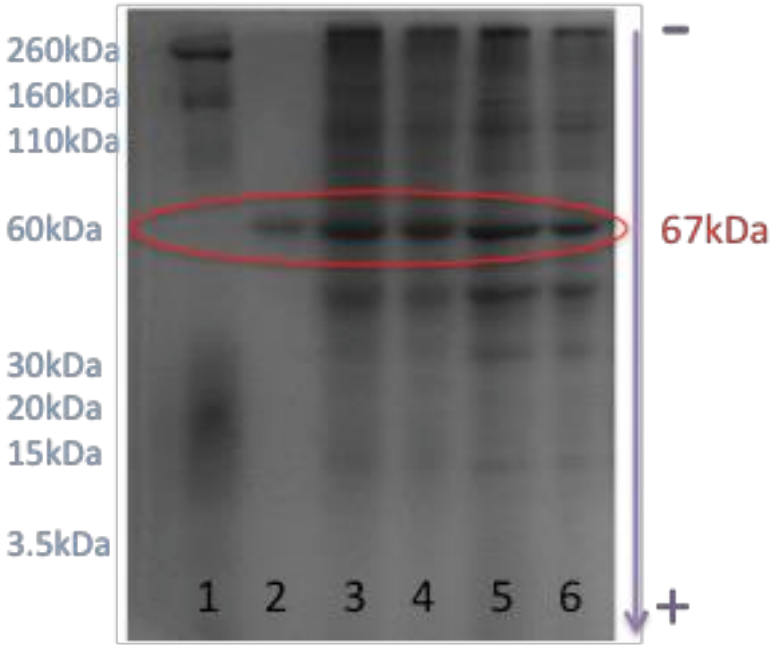

Fig. 4.33a:- Comassie stained gel highlighting extracted proteins from platelets. All platelets were treated before being lysed and 40 µg of protein per lane was loaded onto a SDS gel. Highlighted bands indicate a size of 67 kDa. 1: An appropriately sized prestained marker, 2: Nitrated HSA, 3; Untreated platelets, 4: Platelets treated with Sin-1, 5: Platelets treated with 10 % (v/v) AGE, 6: Platelet proteins treated with both Sin-1 and 10 % (v/v) AGE (final concentrations).

A second gel of electrophorectically separated proteins were transferred to a Polyvinylidene fluoride (PVDF) membrane and probed using a monoclonal mouse anti nitrotyrosine antibody (Santa Cruz, USA). The primary antibody was tested for specificity by adding excess 3-nitrotyrosine (Sigma Aldrich, Gillingham, UK) during the primary antibody incubation period, and upon development no bands were observed, suggesting that the antibody was specific for nitrotyrosine.

As the antibody tested positively for nitrotyrosine specificity, the experiments were repeated without the addition of 3-nitrotyrosine during the primary antibody incubation period. Upon development bands with a higher molecular weight of approximately 105 kDa were observed than the 67 kDa that was initially expected. The control and Sin-1 treated platelet extracts displayed no significant difference in the expression of nitrotyrosine, whereas platelet samples treated with 10 % (v/v) AGE had a diminished concentration of nitrotyrosine to the control level of expression. Platelet lysate

samples challenged with both Sin-1 and 10 % (v/v) of AGE appeared to have no expression of nitrotyrosine, as the presence of AGE with Sin-1 has seemingly decreased the concentration of nitrotyrosine exhibited with Sin-1 alone (Fig. 4.33b).

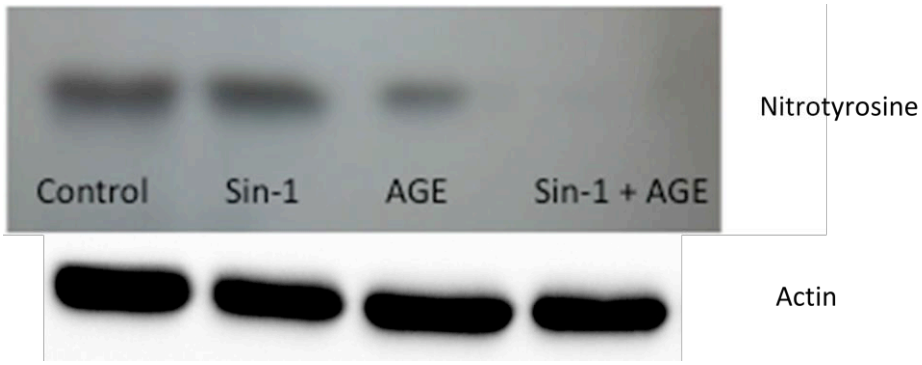

Fig. 4.33b:- Western blot image indicating nitrotyrosine in lysed platelets with different treatments. Untreated platelets compared to positive control Sin-1, 10 % (v/v) AGE, platelets treated with Sin-1 and 10 % (v/v) AGE (final concentrations). All platelets were treated before being lysed and 40 µg of protein per lane was loaded onto a SDS gel, with the actin loading control confirming the same amount of protein per lane. Once separated, proteins were transferred to PVDF membrane before being probed with a nitrotyrosine specific antibody.

Marcondes *et al* (2006) observed a similarly sized band, which through protein purification and analysis was found to be nitrated α-actinin isoform 1. α-actinin is a cytoskeletel protein, which is part of the actin-cross-linking protein family and is involved in the actin network and reorganisation during platelet activation (Marcondes *et al* 2006, Tadokoro *et al* 2011). As AGE may influence platelet α-actinin, (Tadokoro *et al* 2011), the effect of AGE upon the platelet actin structure and platelet shape change was investigated.

4.4.7 The effect of AGE upon platelet shape change and platelet actin structure

4.4.7.1 Does AGE affect ADP induced platelet shape change?

Upon stimulation with ADP, platelets undergo a series of shape changes that ultimately lead to the formation of an aggregate (Fig. 3.26). To assess whether the inhibition of aggregation induced by AGE affects this process, platelets were imaged using scanning electron microscopy (SEMic) over a time course to monitor the shape change before and during aggregation. SEMic is a type of electron microscopy that images a sample by scanning via a high energy of electrons. Imaging requires the samples to be electrically conductive, after fixation samples are therefore coated with an ultrathin layer of a conductive material, such as gold to enable imaging.

Unstimulated platelets are spherical in shape and once activated with ADP, shape change is initiated. Within the platelet through complex signalling the platelet actin structure is rearranged and pseudopods are formed. Over the time course, the stages of platelet shape change were clearly observed; initially the platelets flatten, becoming stellate in shape and eventually become amorphous and form a clot (Fig. 4.34a).

Results show that AGE can inhibit ADP induced aggregation, therefore it is of interest to observe the effect AGE has upon platelet shape change (Fig. 4.34b). Control platelets, unstimulated at 0 seconds, are visibly present in their discoid inactive shape. At 10 and 30 seconds, there is some aggregation; however the majority of the platelets remain inactive. At 60 seconds, despite the number of platelets visible, they still remain in their inactive discoid shape.

When compared to the control sample (Fig. 3.26), the AGE treated platelets clearly display inhibition of ADP induced aggregation across the entire time course, showing a decreased presence of filopodia. Upon activation of platelets, pseudopods should begin to form, indicating the disassembly of the actin cytoskeleton initiating shape change within the platelet. AGE treated platelets fail to show the same prevalence of filopodia, indicating that the majority of platelets are inactive despite the addition of ADP (Fig. 4.34).

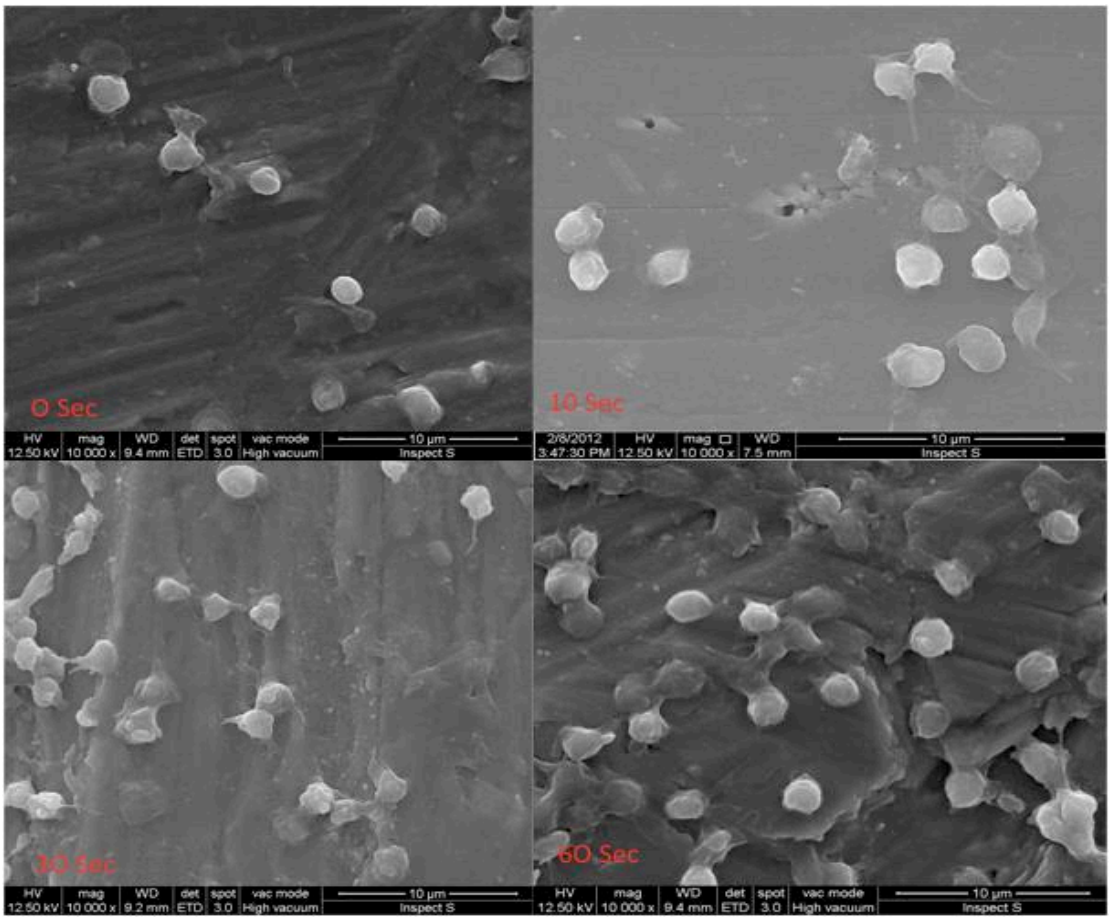

Fig. 4.34:- SEMic images indicating the effect of platelets treated with AGE upon ADP induced activation. Platelets were first treated with 10 % (v/v) AGE (final concentration) before aggregation was initiated with ADP. Images indicate platelets before aggregation (0 sec), and at time periods of 10 sec, 30 sec and 60 sec after aggregation was initiated.

4.4.7.2 Platelet actin structure after treatment with AGE

The formation of filopodia in platelets upon activation requires the reorganization of the platelet cytoskeleton, more specifically platelet actin. To observe the change in actin structure, platelets were fixed, permeabilized and labeled with a monoclonal fluorescently FITC labeled actin specific antibody (Sigma Aldrich).

Control unstimulated platelets at time point 0 seconds are observed to be discoid in shape and show no protruding pseudopodia. Once activated with ADP, filipodia can clearly be observed as well as the change in shape of the platelet before ultimately at 60 seconds, an aggregate is formed containing multiple platelets (Fig. 3.29).

Platelets treated with AGE remain in their inactive discoid shape across the time course with few filopodia visible, even 60 seconds after ADP activation. Despite the high amount of inhibition of platelet aggregation which is normally observed after treatment with 10 % (v/v) AGE, there was a small aggregate present at 60 seconds after activation. This therefore indicates AGE does not induce 100 % inhibition of platelet activation. Aggregation studies with AGE, induced approximately 90 % inhibition of platelet aggregation, fluorescent imaging of the actin cytoskeleton also corroborates this percentage, as a very small amount of activated platelets are visible in AGE treated platelet samples (Fig.4.35).

Despite the small amount of activation, it can clearly be observed that AGE can inhibit ADP induced activation and the reorganisation of platelet actin cytoskeleton as platelets remain in their inactive state even after stimulation with ADP (Fig.4.35).

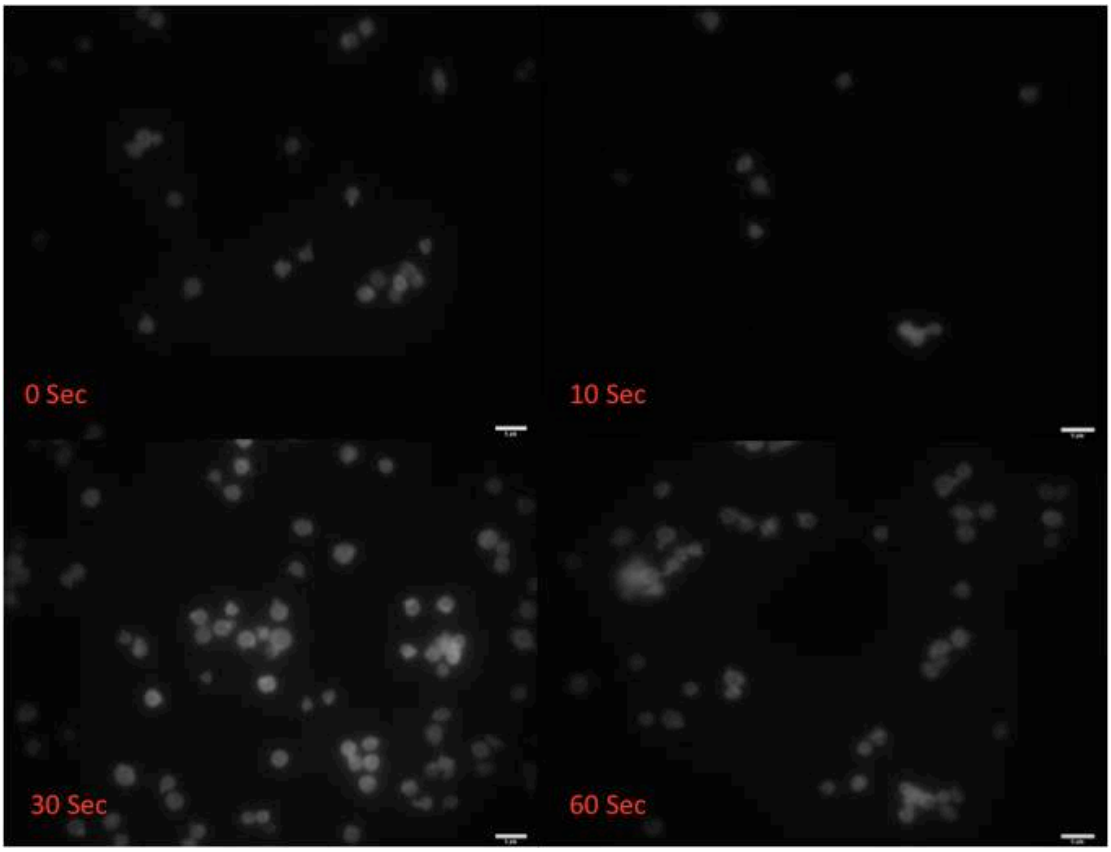

Fig. 4.35:- The effect of AGE upon platelet actin after shape change initiated by ADP. Fluorescently labeled images of platelet actin at 100x. Platelets were treated with 10 % (v/v) AGE and indicate fluorescently actin labeled platelets before aggregation (0 sec), then at time periods of 10 sec, 30 sec and 60 sec after aggregation was initiated with ADP.

AGE inhibits ADP induced platelet shape change and prevents the reorganisation of the actin cytoskeleton. The activation of platelets is reliant upon inside-out signalling that is mediated by specific integrins such as the fibrinogen receptor GPIIb/IIIa. The binding of fibrinogen is the last step in the aggregation process in all agonist induced platelet aggregation. The expression of integrins and inside-out signalling are associated with cytoplasmic proteins such as actinin, as they provide scaffolding within the cell, connecting signalling pathways to the cytoplasmic network (Tadokoro *et al* 2011). As AGE inhibits ADP induced platelet aggregation and prevents actin re-organsation, the binding of platelets to fibrinogen should also be inhibited.

4.4.8 The effect of AGE on platelet adhesion to fibrinogen

The binding of fibrinogen is an important step in the full aggregation of platelets *in vivo*. The expression and the change from low to high affinity of the integrin GPIIb/IIIa mediates inside-out signalling and is responsible for binding fibrinogen.

AGE can inhibit fibrinogen binding by decreasing the expression of GPIIb/IIIa (Allison *et al* 2012), whereas NO can inhibit the binding of platelets to fibrinogen both by cGMP dependent and independent mechanisms (Oberprieler *et al* 2007, Cardoso *et al* 2010). To investigate the effect of AGE compared to NO, and whether the inhibition of fibrinogen binding is cGMP linked, platelets were treated with Sin-1 and final concentrations of AGE between 0.78 % - 10 % (v/v).

The effects of AGE upon fibrinogen binding were investigated using a simple assay utilizing immobilized fibrinogen. The inhibitor 4-[4-[4-(Aminoiminomethyl) phenyl]-1-piperazinyl]-1-piperidineacetic acid trihydrochloride (GR144053) prevents fibrinogen binding by inhibiting the fibrinogen receptor GPIIb/IIIa. Platelets were treated with either PBS for the positive control or GR144053 as the negative control. Other platelets were challenged with either Sin-1, AGE with concentrations between 0.78 % - 10 % (v/v) or with Sin-1 plus 10 % (v/v) AGE. The binding of resting platelets (Fig. 4.36) and ADP activated platelets (Fig. 4.37) to fibrinogen was measured. The binding of resting platelets to the immobilised fibrinogen will provide an indication of the amount of spontaneous binding (Fig. 4.36).

Resting platelets displayed minimal binding to immobilized fibrinogen. Platelets that were treated with GR144053 displayed similar levels of binding as untreated resting platelets, indicating the negligible amount of spontaneous platelet binding to fibrinogen. Despite this, platelets treated with Sin-1, 1 % (v/v) AGE, 10 % (v/v) AGE and Sin-1 plus 10 % (v/v) of AGE all exhibited a significant decrease in fibrinogen binding when compared to the control data (Fig. 4.36).

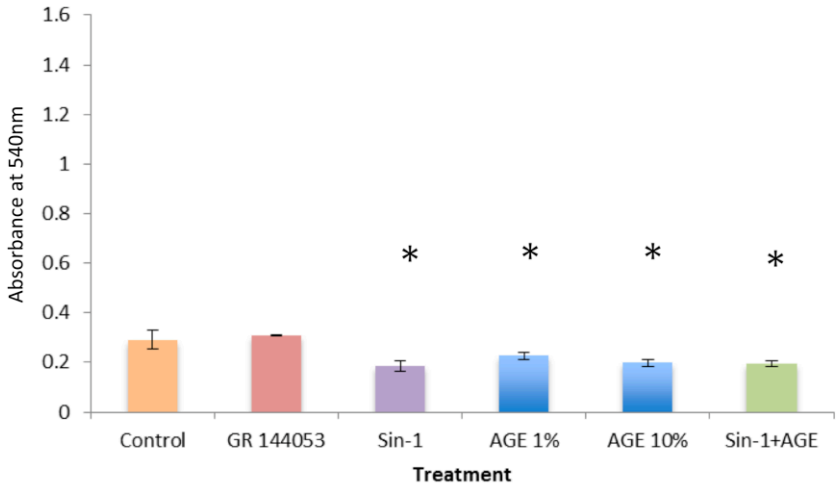

Fig.4.36:- Binding of resting platelets to immobilised fibrinogen. The binding of resting platelets ± SEM to immobilised fibrinogen. Platelets were either untreated (Control), treated with GR144053, Sin-1, 1 % (v/v) AGE, 10 % (v/v) AGE, or both Sin-1 and AGE 10 (v/v) % (final concentrations), before being added on the fibrinogen coated plate. Statistical analysis calculated to untreated platelets (Control) via students t test and indicated via * over respective point with $p \leq 0.05$ (n=6).

ADP activated platelets (Fig. 4.37) displayed a substantial increase in the overall percentage of binding to fibrinogen when compared to resting platelets (Fig. 4.36). Activated platelets treated with GR144053 exhibited the same proportion of binding as in resting platelets, suggesting the inhibitor is successfully bound to the GPIIb/IIIa receptor.

Platelets challenged with Sin-1 before activation was induced with ADP, significantly decreased fibrinogen binding in comparison to the control data. Activated platelets treated with AGE displayed a similar dose dependent pattern of inhibition in fibrinogen binding (Fig. 4.37) that was also observed in aggregation studies (Fig.4.1). At 0.78 % (v/v) AGE there is no significant effect upon activated platelet adhesion to

fibrinogen compared to the control data. Platelets challenged with AGE from concentrations 1.56 % to 10 % (v/v), however displayed a significant decrease in platelet to fibrinogen adhesion when compared to the untreated control platelets. Platelets that were treated with either Sin-1 or 10 % (v/v) AGE significantly decreased fibrinogen binding. However, activated platelets that were treated with both Sin-1 and 10 % (v/v) AGE exhibit an interesting result, as compared to Sin-1 alone, fibrinogen binding has decreased, however contrastingly compared to 10 % AGE alone platelet adhesion to fibrinogen has increased (Fig. 4.37).

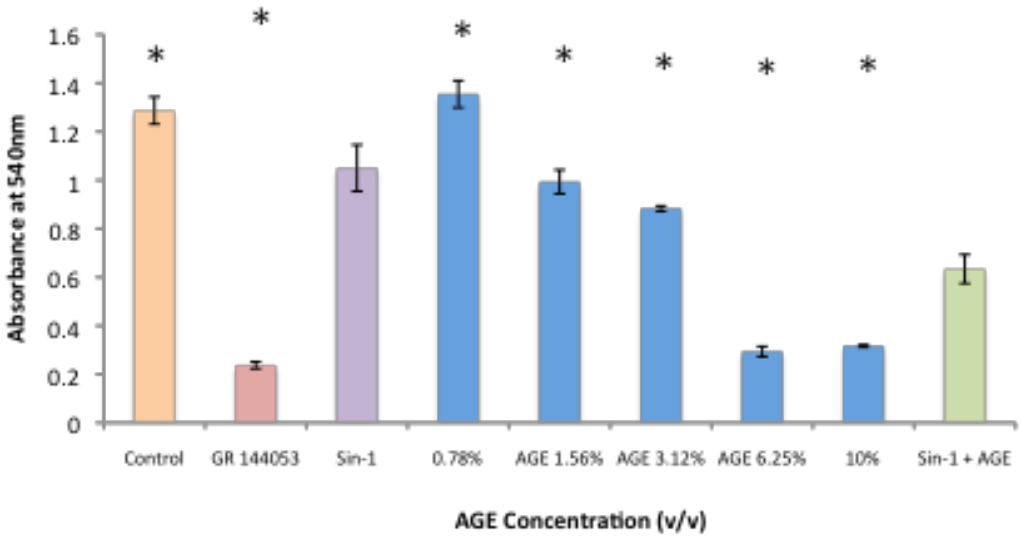

Fig. 4.37:- The binding of ADP activated platelets to immobilized fibrinogen. Binding of ADP activated platelets to a fibrinogen coated 96 well plate ± SEM. Platelets were untreated (Control) or treated with GR144053, Sin-1, AGE with final concentrations between 0.39 %-10 % (v/v), or both Sin-1 and 10 % (v/v) AGE before platelet aggregation was initiated by ADP on the fibrinogen coated plate. Statistical analysis calculated to untreated platelets (Control) via students t test and indicated via * over respective point with $p \leq 0.05$ (n=6).

4.5 Discussion

Having established correct functionality within the platelet signalling system using the chemical inhibitors Sin-1, PGE_1, IBMX, ODQ, SQ 22536 and Zaprinast (Chapter 3), the effect of AGE on the biochemical signalling pathways was thoroughly investigated.

4.5.1 The effect of AGE upon ADP induced platelet aggregation

To corroborate previous findings, the effect of AGE upon platelet aggregation was re-established. As expected, AGE was observed to inhibit ADP induced platelet aggregation in a dose dependent manner (Fig.4.1) with concentrations of AGE at 10 % (v/v) and above causing maximal inhibition (Fig. 4.2, Rahman and Billington 2000, Allison *et al* 2006a).

Herbal extracts have previously demonstrated the capability to influence platelet cGMP (Pierre *et al* 2005). As AGE can increase cAMP, it is likely that AGE will also affect cGMP, however the effect of AGE upon platelet cGMP is currently unknown. It is widely known that NO stimulates the synthesis of cGMP, therefore the NO donors Sin-1 and SNP were utilized as positive controls for comparable data to help elucidate the effect of AGE upon the cyclic nucleotide (Cardoso *et al* 2010, Fig. 4.3).

Both Sin-1 and SNP inhibit platelet aggregation by increasing intraplatelet cGMP (Radziszewski *et al* 1995, Crane *et al* 2005) and when combined with either NO donor, AGE caused an increase in the inhibition of platelet aggregation (Fig.4.3). Sin-1 produces both NO and peroxynitrite (Fisch *et al* 1995). To demonstrate that the peroxynitrite from Sin-1 had a minimal effect upon aggregation, a second NO donor SNP was used, which only produces NO (Cavallini *et al* 1996). Both Sin-1 and SNP in combination with AGE produced similar percentages of inhibition, thus indicating that peroxynitrite was not having an influential effect upon platelet aggregation.

The increase in inhibition observed with AGE and Sin-1 is indicative of an additive effect by AGE and NO upon platelet aggregation, suggesting both AGE and NO may both be acting upon platelet cGMP.

If AGE enhances the effect of NO upon cGMP, inhibiting sGC thus preventing the synthesis of cGMP, a reversal of the inhibition of aggregation caused by AGE should be

observed. The sGC inhibitor ODQ, can prevent an increase in cGMP by Sin-1 (Crane *et al* 2005) indicating the inhibitor functions correctly (Fig. 3.6, Fig. 3.14). If the inhibition of aggregation by AGE and NO is cGMP dependent, the presence of ODQ with AGE would reverse the inhibition of aggregation. A reversal of inhibition was not observed, as results with ODQ and AGE were contradictory. Lower concentrations suggest that ODQ can reverse the inhibitory effects of AGE, however at higher concentrations the opposite is observed, as AGE and ODQ acted to increase inhibition of aggregation (Fig.4.6). Similar contrasting results were also observed when analysing the primary and secondary rates of aggregation (Table 4.1, 4.2). As ODQ fails to remove the inhibition of aggregation induced by AGE, this suggests that AGE is influencing other targets within the platelet.

In previous studies, AGE has been shown to increase intraplatelet cAMP (Allison *et al* 2006a), to further investigate the effect of AGE upon cyclic nucleotide signalling, the effect of AGE upon the enzyme AC was established. If AGE inhibits aggregation through cAMP, using the AC inhibitor SQ 22536 to prevent synthesis of cAMP should reverse some of the inhibitory effects of AGE on platelet aggregation. SQ 22536 was able to reverse and prevent stimulation of cAMP by PGE_1 (Fig. 3.8), thus indicating the inhibitor functions correctly (Salzman *et al* 1978, Hardy *et al* 2004). In combination with AGE, SQ 22536 produced contrasting results when analysing total percentage aggregation and the rates of aggregation (Fig. 4.7, Table 4.3, 4.4). The significant differences observed between the rates of aggregation suggest that SQ 22536 is affecting the influence of AGE upon platelets and that AGE may be influencing both cyclic nucleotides simultaneously. To investigate this further aggregation studies were conducted in the presence of both the inhibitors SQ 22536 and ODQ for AC and sGC respectively.

Results obtained when using either inhibitor separately suggest that AGE is influencing the opposite cyclic nucleotide. To identify whether this is the case, both ODQ and SQ 22536 were used together.

Investigations using ODQ and SQ 22536 together displayed similar contrasting results that were obtained when using either inhibitor singularly. These results suggest that AGE may have another target within platelets or that the presence of AGE is influencing the binding of the inhibitors to their respective active sites.

Interestingly when comparing the rates of aggregation of AGE alone to AGE plus ODQ and SQ 22536 (Table 4.5, Table 4.6) nearly all concentrations of AGE displayed an

increase in the primary rates of aggregation with ODQ and SQ 22536 present, however only 0.78 % (v/v) AGE was statistically significant. This suggests that despite the lack of reversal of inhibition of platelet aggregation, the presence of the inhibitors is having an effect. After stimulation with ADP, to achieve full aggregation, the degradation of intraplatelet cyclic nucleotides occurs through respective PDE (Jin *et al* 1998, Schwarz *et al* 2001, Mo *et al* 2004). In the presence of AGE, cAMP (Allison *et al* 2006a) and cGMP may be increased; AGE may therefore act to slow the rates of aggregation through increasing either cyclic nucleotide. In the presence of ODQ and SQ 22536, the primary rates of aggregation are increased, suggesting that AGE cannot fully stimulate cAMP and cGMP due to the inhibition of sGC and AC by the inhibitors ODQ and SQ 22536 (Fig. 4.8, Table 4.5, 4.6).

Platelet PDE are an important part of cyclic nucleotide signalling, as the enzymes are responsible for the regulation of the concentrations of each nucleotide (Essayan 2001, Feijge *et al* 2004, Francis *et al* 2010). If AGE acts to increase platelet cyclic nucleotides, the presence of a PDE inhibitor should further inhibit aggregation, as with no PDE both cAMP and cGMP will be allowed to accumulate inhibiting aggregation further (Francis *et al* 2010). Both the inhibitors IBMX, a non-specific PDE inhibitor (Essayan 2001), and Zaprinast, a PDE5 inhibitor specific for cGMP (Dong *et al* 1995, Dunkern and Hatzelmann 2005), in the presence of AGE increased inhibition of platelet aggregation (Fig. 4.9, Fig. 4.10) This increase in inhibition was expected as AGE can increase cAMP (Allison *et al* 2006, Allison *et al* 2012) and cGMP.

4.5.2 The effect of AGE upon platelet cyclic nucleotides

Cyclic nucleotide signalling within platelets has a key role in regulating platelet activation (Schwarz *et al* 2001). An increase in either cAMP or cGMP will potently inhibit aggregation through activating downstream molecules such as PKA, PKG and preventing the release of intracellular Ca^{2+} stores (Francis *et al* 2010). Degradation of either nucleotide is therefore crucial for platelet aggregation to occur (Purvis *et al* 2008).

Aggregation studies using Sin-1 or ODQ combined with AGE suggest intraplatelet cGMP may be a target of AGE (Fig. 4.2, 4.6). To investigate this hypothesis intraplatelet

cGMP was quantified in the presence and absence of ODQ after treatment with 5 % (v/v) AGE. As platelet cGMP is present in such small concentrations, the nucleotide was quantified both in the presence and absence of IBMX. The presence of IBMX will allow cGMP to accumulate, thus allowing the full effect of AGE to be quantified, whereas without IBMX, the effect of AGE on cGMP with fully active signalling pathways can also be investigated.

In resting platelets treated with IBMX, 5 % (v/v) AGE significantly increased cGMP and ODQ reversed the increase (Fig. 4.11). However this was not observed in ADP activated platelets, as ODQ failed to revert cGMP concentrations back to basal concentrations, indicating AGE is still influencing cGMP (Fig. 4.12). The same pattern is observed in ADP activated platelets with no IBMX pretreatment, however overall concentrations of cGMP are significantly decreased, as expected, due to the active platelet PDE (Francis *et al* 2010). Platelets treated with both 5 % (v/v) AGE and ODQ show an increase in cGMP compared to ODQ alone, and a decrease in cGMP compared to AGE alone data (Fig. 4.13). This corresponds with aggregation data that indicates ODQ does not fully reverse the inhibitory effects of AGE on aggregation. Conflicting data in both aggregation studies and quantifying cGMP, suggest that the presence of 5 % (v/v) AGE may be affecting the binding of the inhibitor ODQ to its active site on sGC. There is no current evidence to help elucidate whether herbal extracts can influence the binding of chemical mimetics, therefore to investigate this further, a lower dosage of AGE was used and the experiments repeated.

In resting platelets pretreated with IBMX, 1 % (v/v) AGE significantly increased control cGMP concentrations and the presence of ODQ reversed this increase (Fig. 4.14). ADP activated platelets samples treated with 1 % (v/v) AGE also showed a significant increase in cGMP compared to the control. This increase was substantially reversed in the presence of ODQ, but does not equate to intraplatelet cGMP with ODQ alone (Fig. 4.15). These results indicate that ODQ can reverse the effect of AGE in resting platelets, however ODQ has little effect in ADP activated platelets, corroborating results obtained in aggregation studies.

There are studies that suggest that increasing one cyclic nucleotide may influence the concentrations of the other (Schwarz *et al* 2001, Feije *et al* 2004). To investigate the effect of AGE upon both cyclic nucleotides, cAMP was also quantified in the presence and

absence of its respective inhibitor SQ 22536. To ensure comparable data to cGMP, intraplatelet cAMP was also quantified in the presence and absence of IBMX.

In resting platelets treated with IBMX, basal intraplatelet cAMP is significantly increased after treatment with 5 % (v/v) AGE, however the AC inhibitor does not reverse this increase in cAMP induced by AGE (Fig. 4.16). AGE at 5 % (v/v) significantly increased cAMP and this increase remains despite the addition of SQ 22536 (Fig. 4.17). Increase in cGMP can affect cAMP concentrations due to cGMPs influence of PDE3 (Schwarz *et al* 2001, Feije *et al* 2004), however the presence the PDE inhibitor IBMX in these studies means this cannot occur. As similar results were obtained in ADP activated platelets with IBMX (Fig. 4.18) it is therefore possible that SQ 22536 is being influenced by AGE therefore as 1 % (v/v) AGE was also investigated. Irrelevant of the activation state or the presence of IBMX, AGE at a concentration of 1 % (v/v) does not significantly increase cAMP, however the presence of SQ 22536 with AGE does significantly decrease cAMP compared to AGE alone (Fig. 4.19 -4.21).

There is evidence to suggest that platelet cGMP may be able to influence platelet cAMP concentrations (Haslam *et al* 1999, Schwarz *et al* 2001) and there are two hypothesized mechanisms by which this can occur. One theory is that platelet PDE, specifically the control of PDE 3 by cGMP, can effect cAMP concentrations (Fisch *et al* 1995, Maurice 2005). Contrastingly another postulated mechanism is that cGMP increases cAMP through the phosphorylation of PKA and PKG (Pelligrino and Wang 1998, Francis *et al* 2010).

Despite inhibiting sGC or AC in the presence of AGE, there remains inhibition of aggregation as well as fluctuations in either nucleotide. To investigate whether AGE can effect one nucleotide via the other, both platelet sGC and AC were inhibited before platelets were challenged with 1 % (v/v) AGE.

Platelet cGMP was only significantly increased by 1 % (v/v) AGE in resting platelets in the presence of IBMX. This increase in cGMP is reversed in the presence of ODQ and SQ 22536 (Fig. 4.22). In activated platelets cGMP is not significantly increased after treatment with AGE. The presence of both inhibitors ODQ and SQ 22536 does however, substantially decrease cGMP concentrations compared to AGE alone treated platelets, irrelevant of pre-incubation with IBMX (Fig. 4.23, Fig 4.24).

The same is observed when quantifying platelet cAMP after incubation with ODQ and SQ 22536 before challenging with AGE in both resting and activated platelets. Intraplatelet cAMP is significantly decreased after incubation with AGE due to ODQ and SQ 22536 regardless of the presence of IBMX (Fig. 4.25, Fig. 4.26, Fig. 4.27).

These results indicate that AGE inhibits platelet aggregation by influencing both platelet cyclic nucleotides, with the majority of the mechanism of inhibition attributed to an increase in intraplatelet cAMP. Results suggest that AGE only moderately influences platelet cGMP, with observations indicating that the increase in cGMP mediated by AGE only further acts to increase cAMP concentrations. As other evidence indicates cGMP may affects cAMP through PDE 3 (Fisch *et al* 1995, Francis *et al* 2010), it is possible that AGE may also use this mechanism.

PGE_1 increases platelet cAMP and when added to pre-aggregated platelets can cause the dispersal of aggregates into single platelets (Fig. 3.23, Puri 1999). As AGE can influence platelet nucleotides, it is likely that AGE will also cause platelets to disaggregate. AGE at all concentrations caused platelet disaggregation (Fig. 4.28). These results suggest that AGE can both inhibit platelets from aggregating and disperse formed platelet aggregates. As platelets have a crucial role in the development of CVD (Projahn and Koenen 2012, Schulz and Massberg 2012), the ingestion of AGE may therefore benefit those at risk of a thrombotic episode and may act to disperse any thrombi already formed.

4.5.3 The effect of AGE upon platelet NO

There are endogenous inhibitors in the cardiovascular system that act to prevent unwanted platelet aggregation and to disperse unnecessary platelet aggregates, such as NO, which is produced by the vascular endothelium (Persson *et al* 2000). *In vivo* NO is hard to directly quantify, therefore metabolites such as nitrite provide an insight into NO activity (Pacher 2007). For instance there is evidence to show that metabolites such as nitrite and nitrate can be converted back into NO under conditions such as hypoxia to promote cell survival (Lundberg and Weizberg 2005).

To investigate the effect of AGE upon platelet NO, nitrite concentrations were analysed in platelet lysates and media using the Griess assay. As AGE has a dark colour and the Griess assay uses colourmetric analysis, a colourless diethyl extract was also analysed.

Platelet lysates alone displayed results close to and under the 3 μM detection limit of the Griess assay, therefore cannot be considered accurate (Sun *et al* 2003, Fig. 4.29). Nitrite concentrations in both the lysate and media treated with Sin-1 displayed an expected increase in the amount of nitrite present in the sample. The addition of 10 % (v/v) AGE to the sample, had no effect upon control nitrite concentrations, however when combined, Sin-1 and AGE displayed a slight increase when compared to the control. A substantial decrease in nitrite was however observed when compared to Sin-1 alone. This suggests that AGE maybe 'mopping' up nitrite, caused by the scavenging of NO free radicals by AGE, as AGE has previously demonstrated antioxidant capabilities (Dillon *et al* 2003) (Fig.4.30).

There is a noticeable difference between AGE and DEA and this could be due to several possibilities. The results using DEA lie on the limits of detection of the assay and therefore could be considered inaccurate. DEA was used only as an analytical tool and will not contain the same components as AGE, therefore may have lost some of the properties of interest when compared to the AGE parent extract. DEA does indicate some scavenging of NO, however due to the assays low sensitivity, this cannot be counted as an accurate depiction of true nitrite concentrations. These therefore may be a misrepresentation of results due to the low sensitivity of this particular assay. (Fig.4.30, 4.31)

As the Griess assay only has a detection sensitivity of 3 μM (Guervera *et al* 1998, Sun *et al* 2003), a commercial fluorescent NO kit was obtained that had greater detection sensitivity. As AGE has previously displayed some auto-flourescence properties, the garlic extract was first tested using a nitrite standard with no platelets present. Both AGE and the colourless extract exhibited obscure negative results, therefore due to these results the fluorescent assay could not be utilised for further experiments using platelet samples (Fig. 4.32).

RNS can nitrate tyrosine residues creating nitrotyrosine, which can serve as another marker for NO activity *in vivo* (Olas *et al* 2004). The expression of nitrotyrosine is associated with numerous diseased states (Kevil *et al* 2011), including atherosclerosis (Upmacis 2008). Quantifying platelet nitrite indicated that AGE might be scavenging NO,

as nitrotyrosine is another marker of NO activity, the expression of the protein was quantified in lysed platelets after treatment with AGE. A similar result is observed when nitrotyrosine levels in lysed platelets are compared to nitrite concentrations. This protein was identified to be nitrated α-actinin, a structural cytoskeletal protein involved in several processes within the platelet (Marcondes *et al* 2006, Tadokoro *et al* 2011). In the presence of 10 % (v/v) AGE, the levels of nitrotyrosine seemingly decrease, however after treatment with both Sin-1 and 10 % (v/v) AGE they appear to be completely removed, indicating that AGE may be scavenging any present RNS (Fig. 4.33). Other herbal preparations including green tea and red wines have also shown to significantly inhibit the nitration of proteins (Bixby *et al* 2005, Souza *et al* 2008).

The results obtained during investigations of both nitrite and nitrotyrosine levels indicate that AGE may be interfering with NO signalling. This is possibly due to the high quantity of sulphur containing compounds found in AGE. The effect of AGE upon free radicals has previously been identified, with AGE able to scavenge superoxides (Dillon *et al* 2003, Morihara *et al* 2011). SAC, which is present is AGE, has previously been identified to be able scavenge superoxides (Ide and Lau 1999, Cope *et al* 2009). The presence of thiols has also demonstrated the ability to affect the expression of nitrotyrosine (Balabanli *et al* 1999, Olas *et al* 2004). Thus the treatment of platelets with AGE may decrease the presence of the nitrosylated proteins due to thiol containing compounds within AGE and may account for the diminished concentrations of nitrotyrosine within these results.

Quantifying markers of NO indicates that AGE may be scavenging available NO. This is contrasting to the results observed with NO and AGE upon platelet aggregation that suggest there is an additive effect. It is therefore likely that the increase in inhibition of platelet aggregation with NO and AGE is due to increases in either or both cGMP and cAMP. Sin-1, as a NO donor can increase cGMP and has also displayed the ability to increase cAMP in the presence of an AC activator (Fisch *et al* 1995). As AGE can increase intraplatelet cAMP, the garlic extract may thus act as an AC activator. AGE also exerts moderate effects upon intraplatelet cGMP. The increase in inhibition of platelet aggregation observed with Sin-1 and AGE combined may therefore be due to an increase in both cGMP and cAMP, mediated by each reagent.

4.5.4 The effect of AGE upon platelet shape change and adhesion

Increasing intraplatelet cyclic nucleotides can inhibit aggregation and activation (Schwarz *et al* 2001). As the nitration of the cytoskeletal protein α-actinin was also influenced by AGE, platelets were imaged to observe the shape change and the formation of a clot, after treatment with AGE and activation by ADP (Fig. 4.34). Platelets that were challenged with 10 % (v/v) AGE before activation was induced by ADP were also imaged and are timed 0 seconds. Some activation of platelets is observed due to the formation of protruding filopodia, however despite this activation, the majority of the platelets imaged remained spherical in shape and failed to flatten and form an aggregate (Fig. 4.34).

The remodeling of the platelet from its inactive discoid shape to the formation of filopodia and the platelets new shape, involves the destruction of the actin skeleton (Pula and Poole 2008). Remodeling is achieved by an increase in F actin, which is in a polymeric state (Jin *et al* 1998, Jagroop *et al* 2000, Jagroop *et al* 2003, Kudryashov and Reisler 2012). This remodeling of the platelet actin structure can clearly be observed in the control platelets as well as the migration of surrounding platelets to the developing aggregate. A brighter appearance forming leading edge is also observed in platelets forming filopodia. This indicates a higher presence of labeled actin, allowing for platelet shape change and the formation of filopods (Fig. 3.29). This is visibly absent from platelets challenged with 10 % (v/v) AGE. Only a small percentage of platelets treated with AGE were deemed to be in an activated state by the presence of filopodia. Most AGE treated platelets that were imaged, were spherical in shape throughout the time course, unlike the control. AGE treated platelets did not migrate together suggesting the absence of the release of platelet chemotactic factors, a key factor in the recruitment of more platelets (Gachet 2001). At 60 seconds, a small aggregate is present, however as 10 % (v/v) AGE does not promote 100 % inhibition this is consistent with previous results (Fig. 4.35). Images suggest that AGE inhibits the remodeling of platelet actin, thus preventing ADP induced platelet shape change.

Once stimulated, for platelets to form a stable clot, the binding of fibrinogen is an essential step (Bennett *et al* 2009). After platelet activation via ADP, through inside out signalling, the integrin GPIIb/IIIa becomes exposed allowing the binding of fibrinogen (Ohlman *et al* 2000, Nieswandt *et al* 2009). AGE can inhibit ADP fibrinogen binding

through a decrease in the expression of the GPIIb/IIIa complex (Allison *et al* 2012). Fibrinogen binding in both resting and ADP activated platelets was inhibited by concentrations 1.56 % - 10 % (v/v) AGE (Fig. 4.36, Fig. 4.37). The NO donor Sin-1, also inhibits fibrinogen binding and is comparable to 1.56 % and 3.12 % (v/v) AGE, as the increase in cGMP caused by Sin-1 inhibits fibrinogen binding (Nowak *et al* 2002*,* Keh *et al* 2003, Oberprieler *et al* 2007). As AGE can influence intraplatelet cyclic nucleotides it is likely that the decrease in fibrinogen binding is mediated by the effect of AGE on both cGMP and cAMP.

The combination of Sin-1 and 10 % AGE does not promote the same inhibition of binding as 10 % (v/v) AGE alone. This is an interesting observation, as Sin-1 and AGE at all concentrations increased the inhibition of ADP induced platelet aggregation. This may be due a technical error, however as AGE also possesses antioxidant properties (Dillon *et al* 2003), this needed further investigation and was beyond the scope of this thesis.

4.6 Overview of Key Results

- Concentrations above 0.78 % (v/v) of AGE inhibit ADP induced aggregation of isolated human platelets.
- AGE and NO donors combined, significantly increased the inhibition of platelet aggregation, suggesting an additive effect of NO and AGE.
- With PDE inhibited in the presence of AGE an increase in the inhibition of platelet aggregation is observed.
- AGE induced a significant increase in cAMP and had little effect upon cGMP.
- AGE exerts its inhibitory effect on platelet aggregation mainly via an increase in cAMP.
- AGE at a concentration of 5% (v/v) increases intraplatelet cGMP concentrations.
- Platelet cAMP is significantly increased by AGE at a concentration of 1% (v/v) and 5% (v/v), indicating a dose dependent response.
- AGE may enhance potential crosstalk between cGMP and cAMP via specific PDE resulting in higher levels of intraplatelet cAMP.

- Inhibiting AC and sGC does not significantly reduce the inhibition of platelet aggregation induced by AGE. This therefore suggests that AGE may have another target within the platelet and it may be able to increase either cyclic nucleotide via a mechanism independent of the stimulation of AC and sGC.
- AGE at 10% (v/v) decreased the NO markers nitrite and nitrotyrosine. It is possible that AGE is acting as an antioxidant and mopping up the NO radical.
- AGE inhibited both ADP induced shape change and the re-organization of the platelet actin cytoskeleton
- AGE decreased the binding of platelets to fibrinogen hence AGE may reduce the inhibition of platelet aggregation by altering the properties of the GpIIb/IIIa receptor.

Results in this chapter indicate that AGE can inhibit platelet aggregation, with the majority of the mechanism can be attributed to increases in platelet cyclic nucleotides. AGE also intereferes with NO signalling due to scavenging of the free radical, and can influence the binding of chemical inhibitors to their respective active sites.

5 General Discussion

Pharmaceutical intervention for CVD is costly and often has unwanted side effects for the patient. Long-term preventative medication can result in reduced efficacy of the medication and can even lead to drug resistance (Xiang *et al* 2008, Floyd *et al* 2012, Kovacs *et al* 2013). Evidence has suggested that natural compounds rich in polyphenols have a role in reducing the numerous risk factors associated with CVD (Mickalska *et al* 2010, Vasanthi *et al* 2012). The consumption of garlic has been widely investigated (Butt *et al* 2009), especially the supplement AGE termed Kyolic, marketed by Wakunaga Ltd, which has exhibited all of the aforementioned cardioprotective properties (Rahman and Lowe 2006). The inhibition of platelets is vital in the treatment of CVD to prevent further thrombotic events resulting from the occlusion of the vasculature (Projahn and Koenan 2012). AGE has previously shown to inhibit platelet aggregation and establishing the mechanism of inhibition of platelet aggregation by AGE may provide a novel target for pharmaceutical intervention. It may also provide an alternative therapy for treating and preventing CVD that is both economically viable and has fewer side effects.

During this study, several dosages of AGE were investigated from 0.195 % (v/v) to 25 % (v/v) to establish the effect upon ADP induced platelet aggregation. High concentrations of AGE saturated the system, consistently achieving maximal inhibition of platelet aggregation. Concentrations less than 0.78 % (v/v) of AGE had little impact on platelets with the IC_{50} value approximately 3 % (v/v) *in vitro*. An effective dietary dosage of AGE *in vivo* has yet to be established and would form the primary aim of an *in vivo* trial.

ADP is an important agonist and is released from the platelet dense granule system to reinforce and recruit more platelets to the site of injury *in vivo*. ADP was the chosen agonist due the important role it has in platelet aggregation and the various essential signalling molecules involved throughout the ADP pathway. Previous work has identified that AGE targets important signalling molecules in the ADP pathway including cAMP and Ca^{2+} (Allison *et al* 2006). In an *in vivo* situation platelets would be targeted with several other agonists that can induce activation. Previous investigations identified that AGE can inhibit platelet aggregation induced by the agonists ADP, thrombin, adrenaline,

AA and collagen (Allison 2007). An increase in either cAMP or cGMP will inhibit platelet aggregation irrelevant of the initial agonist (Smolenski 2011). The work reported in this thesis has identified that AGE targets platelet cyclic nucleotide signalling and as AGE can influence both cyclic nucleotides, this provides a crucial mechanism of inhibition of aggregation for all agonist induced platelet aggregation. Thus as AGE targets cyclic nucleotides, platelet activation should be inhibited *in vivo* as well *in vitro* irrelevant of the initial agonist.

As these other herb extracts also have an increasing effect upon intraplatelet cyclic nucleotides, resulting in the inhibition of platelet activation, it may be possible to identify key compounds that are responsible for the inhibition of platelet aggregation. This could be achieved by studying the components of AGE, nettles and alfafa extracts to establish whether there are any similar components or structures. Comparing the components of these herbs may provide a key compound responsible for increasing platelet cyclic nucleotides.

As cAMP and cGMP signalling are interlinked, it was of importance to establish the effect of AGE upon platelet cGMP as well as associated NO signalling to elucidate the full inhibitory mechanism exerted by AGE upon platelets (Chapter 4). This particular study was unique, as the effect of AGE upon intraplatelet cGMP and the cross effect of cGMP upon cAMP has not previously been identified.

It is thoroughly documented that NO donors such as Sin-1 and SNP inhibit platelet aggregation by increasing cGMP through the direct stimulation of sGC (Fisch *et al* 1995, Marcondes *et al* 2006). As there is little evidence to suggest the presence of the membrane bound particulate GC, this must therefore occur through stimulation of sGC (Walter and Gambaryan 2009). Platelet aggregation was also inhibited independent of cGMP, using the agonist ADP (Chapter 3, Fig. 3.7, p60). This was established as the inhibitory effects of low concentrations of Sin-1 (8 µM) in the presence of ODQ were reversed, however the presence of ODQ failed to reverse high dosages of Sin-1 (100 µM). This indicates that NO at high concentrations can inhibit platelet aggregation independently of cGMP. These investigations using different concentrations of the NO donor Sin-1, specifically at a high dosage, suggest that platelet aggregation was inhibited independently of cGMP. Previous studies displaying cGMP independent mechanisms used TXA_2 and investigated the binding of platelets to immobilized fibrinogen (Crane *et al* 2005,

Oberprieler *et al* 2007). When investigating cGMP independent platelet inhibition the agonist ADP has not previously been utilised thus making these findings novel (Chapter 3).

This study utilised numerous inhibitors to allow any effect upon one signalling target to be observed. To establish the inhibitors were functioning correctly, each one was tested with a known stimulator of the target. For instance, SQ 22536 prevented increases in concentrations of intraplatelet cAMP, whereas the PDE inhibitor IBMX allowed each cyclic nucleotide to increase, potentiating the inhibition of platelet aggregation through the accumulation of cAMP and cGMP.

Using the inhibitors SQ 22536, ODQ, IBMX and Zaprinast, the effect of AGE on cyclic nucleotide signaling was investigated. Experimentation with high concentrations of AGE suggested that the inhibitors SQ 22536 and ODQ were being displaced from their corresponding active sites. This potential influential effect of high concentrations of AGE has previously not been identified and is a key finding of these investigations, and is important to apply to future investigations when using inhibitors with other natural compounds.

The displacement of inhibitors is not the only technical consideration when working with AGE, as there are other properties of AGE that could interfere with experimentation. For instance AGE has a very dark colour, thus when using a colourmetric assay or methodology, this dark colour may interfere with the results. Steps were taken to ensure that the colour of AGE had little or no impact on results. This included numerous different positive and negative controls per method and a colourless extract of AGE using diethyl ether, however there still remains a possibility of interference due to the dark colour of AGE.

Previous studies indicated that AGE possesses autofluorescent properties. The results of the investigations reported have also highlighted these properties, with the autofluorescence potentially causing interference with the fluorescent NO assay and necessitating extra washes during fluorescent antibody probing. This is another important technical consideration when working with AGE and using fluorescent methodology.

AGE also has antioxidant properties by demonstrating the ability to attenuate oxidative stress as well as enhance NO (Ide and Lau 1999, Morihara *et al* 2002). The antioxidant properties of AGE may possess the ability to scavenge free radicals such as NO and may also contribute to the inhibition of platelet aggregation.

In the presence of an AC stimulator the NO donor Sin-1 can increase cAMP as well as cGMP through a mechanism, which is postulated to be mediated by PDE3 (Fisch *et al* 1995). Sin-1 and AGE caused an additive inhibitory effect upon platelet aggregation. As AGE can increase cAMP and therefore serve as an AC activator, AGE and Sin-1 acting upon both cyclic nucleotides may explain the additive effect. To investigate this further, the opposing stimulatory effect was examined by measuring platelet cGMP after treatment with cAMP stimulator PGE_1, however cGMP concentrations were not affected by PGE_1. This indicated that there may be a cross linking mechanism between cGMP and cAMP, in that an increase in cGMP may also induce a further increase in cAMP. In the presence of AGE it is therefore possible that Sin-1 could increase both cGMP and cAMP, through negative feedback upon platelet PDE3.

AGE can increase intraplatelet cAMP concentrations (Allison *et al* 2006, Chapter 4, Fig. 4.16 – 4.21, p141-146) however, data suggests that AGE has a moderate effect upon intraplatelet cGMP concentration (Chapter 4, Fig. 4.11 - 4.15, p135-140). Inhibiting sGC and AC displayed a small reversal of inhibition of platelet aggregation in the presence of higher dosages of AGE. This reversal however was not consistent across all concentrations or enough to fully attribute the whole mechanism of inhibition of platelet aggregation by AGE acting upon cAMP and cGMP. If AGE was solely acting upon cGMP and cAMP a higher amount of reversal of inhibition would have been observed, however this was not the case (Chapter 4, Fig. 4.8, p127). It is probable that AGE inhibits platelet aggregation through a significant increase in cAMP both directly through AC and via a moderate increase in cGMP due to cGMP competitively binding PDE3.

Despite the presence of AC and sGC inhibitors, large amounts of inhibition of platelet aggregation remained. Another explanation could be that AGE is influencing cAMP through other platelet adenosine receptors such as A_{2A} or A_{2B}. It is proposed that through regulation of cAMP levels, the A_{2A} or A_{2B} receptors for adenosine can inhibit the expression of the ADP receptors $P2Y_1$ and $P2Y_{12}$, as well as platelet aggregation (Yang *et al* 2010). Aggregation results reported in this thesis suggest that AGE may also target these secondary adenosine receptors, as in the presence of SQ 22536 and ODQ there is still significant inhibition of aggregation induced by AGE. Quantifying cAMP however does not support this theory, as cAMP concentrations are decreased in the presence of SQ 22536 and ODQ. This needs further investigation to fully establish the effect of AGE upon platelet adenosine receptors.

Increasing platelet cyclic nucleotides can inhibit many platelet functions including adhesion, early activation signalling molecules such as Ca^{2+} and G protein activation, granule release and aggregation (Smolenski 2012). The release of Ca^{2+} and the reorganisation of the platelet actin structure is a crucial part of platelet activation (Pula and Poole 2008). The G proteins RhoA and Rac1 are regulated by cAMP and cGMP and have functional roles in actin remodeling, the production of filopodia and aggregation (Gratacap *et al* 2001). AGE can induce increases in cAMP and cGMP therefore the inhibition of platelet shape change and actin reorganization that was observed is likely to be due to increases in platelet cyclic nucleotides that are induced by AGE.

The transduction of cyclic nucleotide signalling can affect numerous platelet functions through the phosphorylation of specific substrates by either PKA or PKG. Activation of either PKA or PKG often results in the phosphorylation of the same proteins, and can inhibit the release of Ca^{2+}, actin reorganization and the expression of integrins. It is therefore likely that AGE induces inhibition of platelet aggregation through influencing platelet cyclic nucleotides from which the effect is mediated onto further signalling proteins by the specific isoforms of PKA or PKG. To examine this further, future work would include establishing the activation of both PKA and PKG by measuring the phosphorylation of specific substrates before and after treatment with AGE.

There are other signalling molecules and receptors involved in platelet activation and aggregation that may also be affected by AGE that have not been investigated in this project. These include numerous platelet integrins, other agonist receptors such as PAR1 and PAR4 and mitogen-activated protein kinases (MAPKs). Subgroups of MAPKs include extracellular signal-regulated kinases (ERK), p38 protein kinases and c-Jun NH_2 terminal kinases (JNK) (Flevaris *et al* 2009). ERK, JNK and p38 are important signalling molecules and are involved in the activation of platelets through outside in signalling regulating the expression of specific integrins (Li *et al* 2006, Begonja *et al* 2007). It is thought that ERK is involved with the scaffolding protein RhoA and the serine/threonine protein kinase Raf1 (Robinson and Pitcher 2013). RhoA can be regulated by cGMP and cAMP (Pilz and Casteel 2003), therefore the full involvement of platelet ERK signalling in response to AGE warrants further investigations.

5.1 Summary of the proposed Mechanisms by which AGE inhibits platelet aggregation

Research has indicated that AGE taken as a dietary supplement leads to an inhibition of platelet aggregation (Rahman and Billington 2000, Allison *et al* 2006).

Investigations in this study provide evidence that the mechanism by which AGE inhibits platelet aggregation is due to AGE targeting platelet cyclic nucleotide signalling and possibly several other molecules within the platelet, including intraplatelet Ca^{2+}.

AGE inhibits platelet aggregation through stimulation of both cGMP and cAMP. Complex signalling work identified that AGE only moderately increases cGMP, therefore the majority of the mechanism of inhibition is most likely due to an increase in cAMP. This may be directly through cAMP or through cGMP influencing cAMP concentrations and respective signalling molecules such as platelet PDE3 (Smolenski 2011).

Furthermore, AGE also prevents the binding of platelets to fibrinogen by affecting the expression of the GPIIb/IIIa receptors and preventing platelet shape change. It has previously been highlighted that AGE has strong antioxidant properties, and can scavenge free radicals *in vitro,* whilst also preventing an increase in oxidative stress *in vivo* (Dillon *et al* 2003). The results of studies reported here also suggest that the antioxidant properties of AGE may be able to scavenge the free radical messenger NO.

5.2 Overview of Key Points

- Concentrations higher than 0.78 % (v/v) AGE inhibits ADP induced aggregation of isolated human platelets.
- NO donors Sin-1 and SNP inhibited ADP induced platelet aggregation.
- AGE combined with the NO donors Sin-1 and SNP increased the inhibition of platelet aggregation suggesting an additive effect.
- AGE at 5% (v/v) increases intraplatelet cGMP concentrations.
- Platelet cAMP is significantly increased by 1 % and 5 % (v/v) of AGE indicating a dose dependent response.
- Sin-1 can increase cAMP in the presence of an AC activator.

- PGE_1 has no effect on the intraplatelet cGMP levels.
- Increases in cGMP induced by AGE, appears to be a minor pathway whilst the major pathway of inhibition of platelet aggregation is via cAMP.
- High concentrations of NO donor Sin-1 can inhibit aggregation via a mechanism independent of cGMP. It is possible that AGE may also be able to manipulate this mechanism.
- Inhibiting AC and sGC does not significantly reduce the inhibition of platelet aggregation induced by AGE, suggesting AGE may have another target within platelets.
- AGE at 10% (v/v) decreased the NO markers nitrite and nitrotyrosine potentially by scavenging the any present free radicals, including NO.
- The binding of platelets to fibrinogen was inhibited by the use of GR 144053, an inhibitor of the platelet fibrinogen receptor glycoprotein GpIIb/IIIa.
- AGE decreased the binding of platelets to fibrinogen and may reduce the inhibition of platelet aggregation by altering the properties of the GpIIb/IIIa receptor.
- AGE can inhibit ADP induced platelet shape change.
- The reorganisation of the platelet actin cytoskeleton was also inhibited after platelets were treated with AGE.

5.3 Conclusions

The aims and objectives of this study were to identify the role of NO and cGMP in platelet functions, and to prove that AGE could influence NO signalling and intraplatelet cyclic nucleotides. The investigations in this thesis provide evidence that AGE can increase intraplatelet cyclic nucleotides resulting in the inhibition of platelet aggregation, adhesion and shape change. During experiments AGE was found to interfere with specific chemical inhibitors, which is an important observation, as future work must consider the potential influence of AGE on such chemical mimetics. The effect of AGE upon NO was not fully elucidated in this thesis, results from these studies suggest that AGE can scavenge the free radical, however more work is necessary to substantiate this preliminary observation.

Platelets have an important role in the vascular system, with abnormal activation of platelets implicated in the development of CVD. Evidence provided in this study corroborates previous work, indicating that the regular consumption of AGE could be beneficial in the prevention and treatment of CVD.

5.4 Suggestions for Future Work

5.4.1. Investigation of other possible signalling molecules targeted by AGE

- Directly quantify platelet PDE after treatment with AGE
- Investigate the effect and expression of soluble guanylyl cyclase, adenylyl cyclase and the adenosine receptors A2B and both before and after treatment with AGE.
- Establish the phosphorylation of VASP, RhoA and other PKA and PKG substrates, providing an indication of protein kinase activity in platelet activation and after treatment with AGE.
- Investigate the effect and expression of specific integrins after treatment with AGE.
- Determine the effect of AGE upon platelet ERK signalling.
- Direct measurement of NOS activity both before and after treatment with AGE. This would be identified through the rate of conversion of $_{L}$-arginine to $_{L}$-citrulline. This will give an indication whether AGE has the ability to stimulate NOS.
- Determine the Ca^{2+} flux within platelets in response to AGE and other stimulators, including NO, for comparison.
- Investigate the elasticity of the platelet membrane and the cellular integrity, to determine if the effect of AGE if any upon individual platelets using atomic force microscopy.
- Directly investigate the effect of AGE upon the binding of chemical inhibitors.

5.4.2. Parameters to be considered for any future *in vivo* trial

- Investigate the time and dose-dependent relationship between AGE and its efficiency in reducing platelet aggregation through determining the minimum time and dose of AGE required that would inhibit platelet aggregation *in vivo*.
- Measurement of the endothelial function by flow mediated dilation, which assesses conduit vessel NO-mediated e*ndothel*ial function particularly that of the brachial artery. AGE will be ingested by human volunteers and the following parameters assessed;
    - Blood flow
    - Conduit and resistance vessel assessments
    - Microvessel function assessment
    - Cardiac structure and function
- The following cellular factors will also be looked at after the ingestion of AGE during the small trial;
    - cGMP concentrations after the ingestion of AGE
    - Nitrite and nitrotyrosine concentrations will be measured to provide an indication of NO activity
    - PGI_2 / TXA_2 levels will be monitored before and after AGE ingestion.

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Appendices

Appendix I

Volunteer Venepucture Consent Form

Volunteer Consent Form:- Blood Donation

Title of Research Project: The Role of Reactive Nitrogen Species and Aged Garlic Extract on Platelet Function

Please initial

1. I confirm that I have read and have understood the information provided [DATE] for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I agree to my blood being taken for use in this research

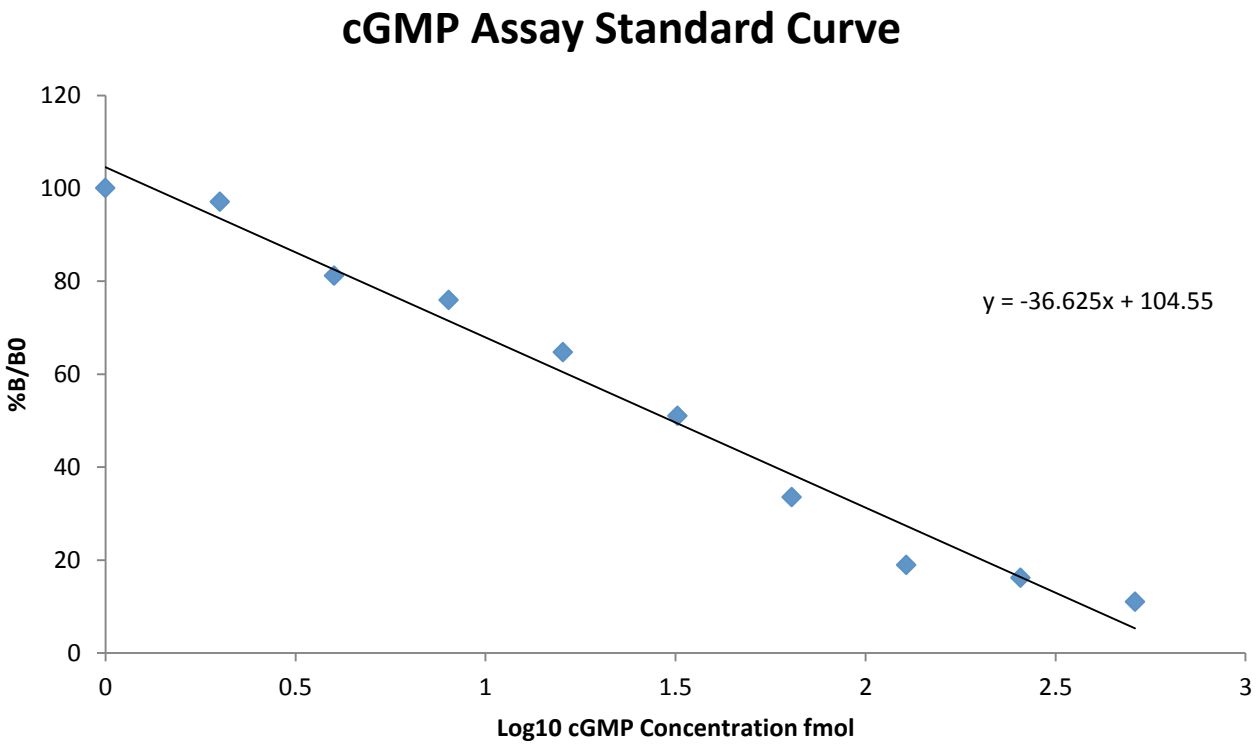
Participant Name	Date	Signature
Researcher	Date	Signature

The contact details of lead Researcher (Principal Investigator) are:

Sarah Smith
Lab 255
Max Perutz Building,
Byrom Street,
Liverpool
L3 3AF
s.smith11@2008.ljmu.ac.uk

Appendix II

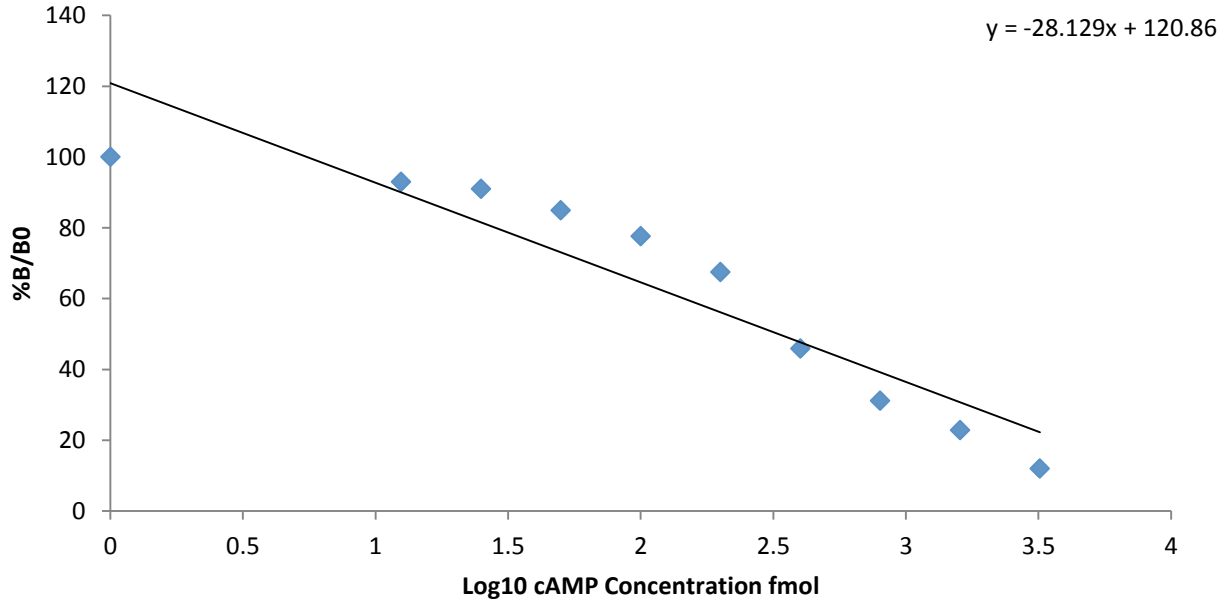
Standard Curve for cGMP EIA


cGMP Assay Standard Curve
120
100
80
60
40
20
0
%B/B0
0
0.5
1
1.5
2
2.5
3
Log10 cGMP Concentration fmol
y = -36.625x + 104.55

Appendix III

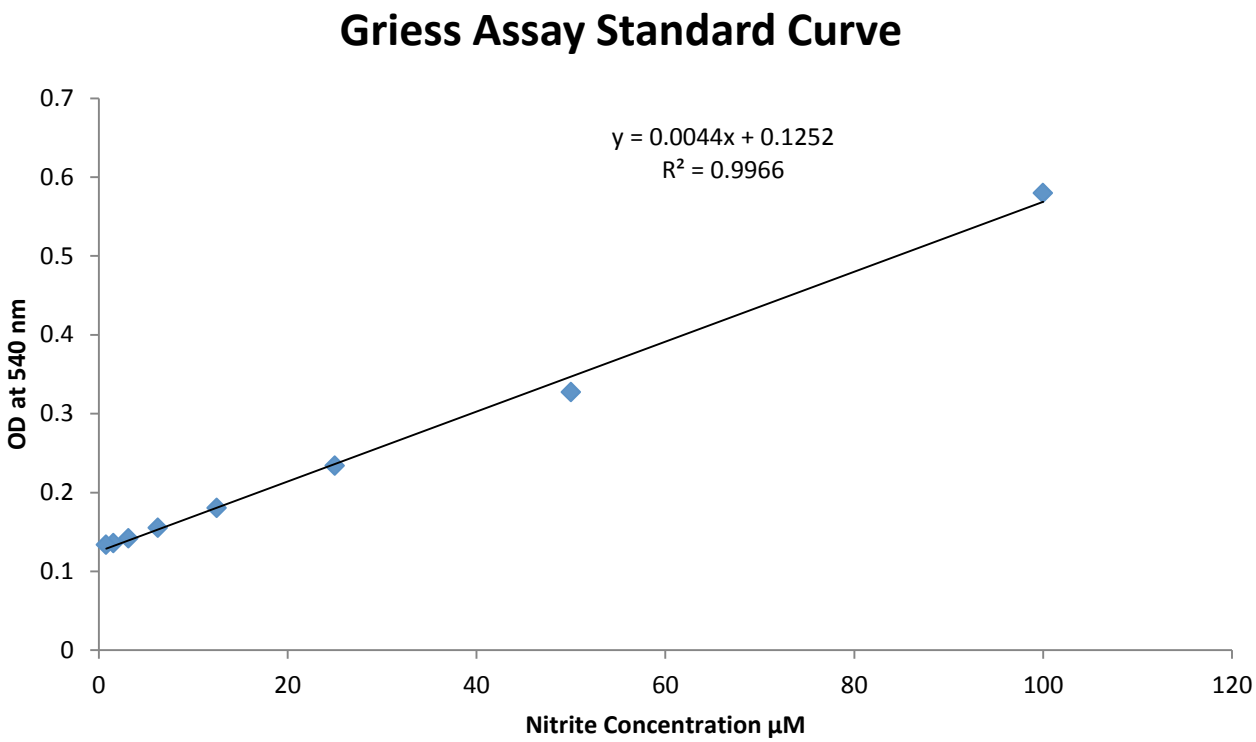
Standard Curve for cAMP EIA

cAMP Assay Standard Curve



Appendix IV

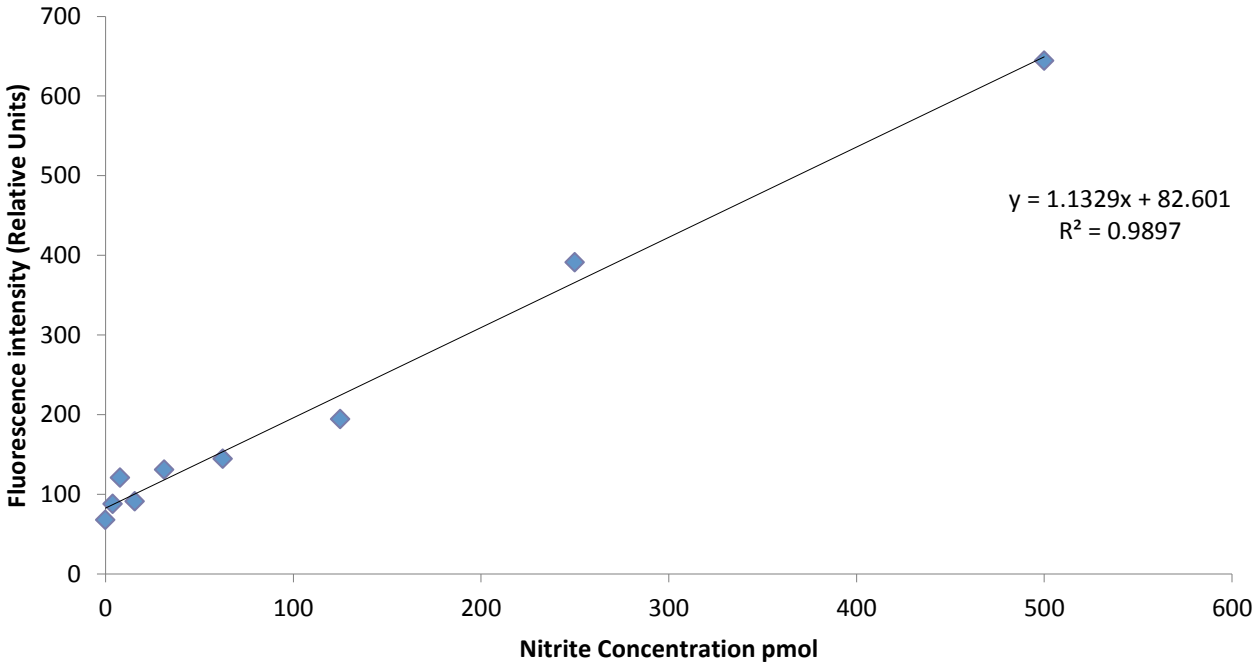
Standard Curve for Griess Assay


Griess Assay Standard Curve
y = 0.0044x + 0.1252
R² = 0.9966
OD at 540 nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
Nitrite Concentration µM

Appendix V

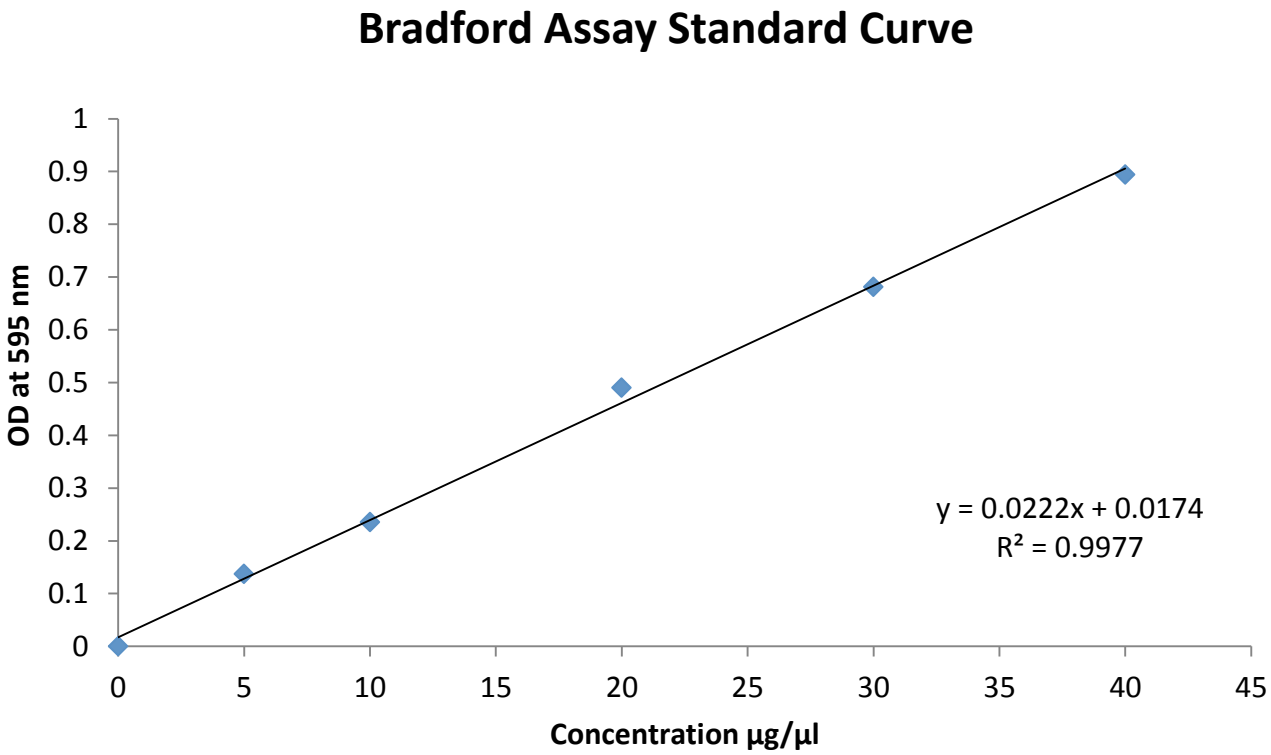
Standard Curve for Fluoroscent NO Assay

Nitric Oxide Fluorescent Assay Standard Curve



Appendix VI

Bradford Assay Standard Curve for determining protein concentrations


Bradford Assay Standard Curve
OD at 595 nm
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
Concentration µg/µl
y = 0.0222x + 0.0174
R² = 0.9977

Appendix VII

Published Paper

Aged Garlic Extract: A possible preventative therapy for thrombotic events

Journal of Food Science and Technology, 2011, 25, 4, p26-27

Aged garlic extract: a possible preventative therapy for thrombotic events

Sarah Smith won the Postgraduate Prize at the 2011 IFST Northern Branch Young Scientists Research Competition; this feature is based on her presentation

Aged garlic extract

There is mounting evidence that the consumption of specific foods can have numerous health benefits. Most of these foods are fruit and vegetables that often contain phytochemicals and can be beneficial in various disease situations, including cancer, cardiovascular disease (CVD), strokes and other thrombotic events. Garlic (*Allium sativum*) is one such phytochemical source and is best characterised for its strong antioxidant properties and a beneficial role in both preventing and treating CVD (1). Commercially, there is a vast array of supplements relating to garlic. One such preparation is aged garlic extract (AGE), which is prepared by soaking sliced raw garlic in 15-20% aqueous ethanol for up to 20 months at room temperature. This extract is filtered and concentrated under reduced pressure at low temperatures and is marketed in both dry and liquid forms under the commercial name Kyolic. This process causes an increase in the concentration of sulphur-based and water-soluble compounds. S-Allylcysteine, a major sulphur compound, is used to standardise AGE (2).

Work over the past 20 years suggests that AGE helps to reduce certain risk factors that lead to CVD. It was demonstrated that AGE helps reduce blood pressure, plasma cholesterol and triglycerides in hyperlipidaemic subjects (1, 3). One major complication of CVD is a thrombotic episode, in which platelets have an extensive role. There is considerable evidence that AGE can inhibit platelets from aggregating in both healthy and in those with CVD (4).

Cardiovascular complications are the leading cause of death within the Western world (5). Restricted blood flow due to the presence of a plaque or plaque rupture can activate circulating platelets leading to thrombotic events.

Platelets

Platelets are a crucial component in haemostasis and can therefore have an important role in obstructive vascular events. In a non-diseased system, vascular endothelial cells produce the gaseous signalling molecule, nitric oxide (NO). Circulating NO can prevent surrounding platelets from aggregating by inducing intraplatelet 3′,5′ guanosine monophosphate (cGMP). In those with CVD, circulating endothelium produced NO is decreased, therefore increasing the chance of platelets aggregating spontaneously.

Two secondary messengers, cAMP and cGMP, are important regulators in platelets. When stimulated, an increased concentration of cAMP or cGMP within the platelet inhibit further activation events and inhibit platelet aggregation. These are very important pharmacological targets to modify platelet activity.

In atherosclerotic candidates (Fig.1), a dysfunctional vascular endothelium does not produce sufficient NO, therefore increasing the likelihood of unwanted platelet aggregation and the potential formation of thrombi or plaque. In this situation there is less intraplatelet cGMP. This is only one contributing factor to a complex pathogenesis of cardiovascular diseases.

In a diseased or damaged vascular state, less NO is produced and is circulated amongst other factors, allowing intraplatelet cAMP and cGMP to decrease. Platelets are more likely to activate and adhere to the vascular lining eventually creating a plaque or thrombus.

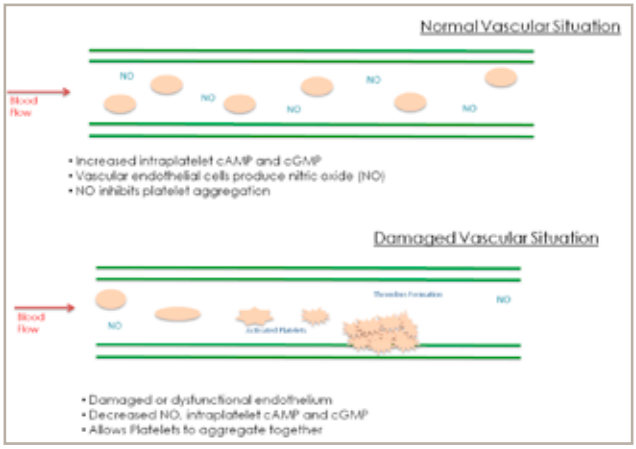


Figure 1. Simplified schematic of both a normal and a damaged/diseased vascular situation. In normal state, vascular endothelial cells produce NO, causing an increase in cGMP in platelets. Intraplatelet cAMP is also higher, prohibiting platelets from aggregating.

Platelet inhibition by aged garlic extract

There have been small-scale clinical trials investigating the benefits of the consumption of this popular garlic supplement and its effect upon platelet biochemistry. Platelets have many agonists that can initiate aggregation and each agonist triggers a different signalling response. Adenosine diphosphate (ADP) is one such agonist and can activate platelets. ADP causes several intracellular responses in

platelets including the inhibition of cAMP production through adenylyl cyclase, the mobilisation of intracellular calcium (Ca^{2+}) stores and a rapid Ca^{2+} influx.

Previous work has shown that AGE can inhibit ADP-stimulated platelet aggregation both *in vitro* and *in vivo* (4). The inhibition observed is due to AGE exerting an effect upon multiple signalling targets within the platelet. One target of AGE is Ca^{2+}. Platelets are reliant upon the mobilisation of intraplatelet stores of Ca^{2+} to be able to aggregate upon ADP stimulation (6). AGE promotes a decrease in intraplatelet Ca^{2+} therefore accounting for part of its inhibitory effect on platelet aggregation (4).

The inhibitory effects of AGE on ADP-initiated platelet aggregation can also be attributed to it acting upon another signaling target. AGE can also increase the levels of intraplatelet cAMP (4) therefore promoting further downstream signaling by this intracellular messenger.

As AGE has an effect upon cAMP, it is possible that AGE might also modify cGMP signalling. Since intraplatelet cAMP is approximately ten-fold higher than cGMP (7), it is possible that the majority of the mechanism of inhibition is due to AGE increasing cAMP rather than cGMP. cGMP acts as secondary messenger much like the other cyclic nucleotide 3′,5′ adenosine monophosphate (cAMP). The membrane-bound enzyme adenylyl cyclase produces cAMP which continues to activate further downstream signalling. Once platelets are activated by the agonist ADP, intraplatelet cAMP and cGMP are decreased allowing aggregation to occur. In resting platelets, levels of cAMP and cGMP are higher than in activated platelets, preventing aggregation from occurring. Once stimulated, both cAMP and cGMP are degraded quickly by their specific phosphodisesterases (PDE), converting them into inactive 5′-nucleotide metabolites, preventing further signalling

As platelets have many agonists that initiate aggregation, with each triggering different signalling responses, it is probable that AGE has several signalling targets within the platelet.

So why take garlic?

Taking a garlic supplement has wide implications in both the treatment and prevention of disease. AGE is beneficial in reducing many of the parameters associated with CVD, including inhibiting platelet aggregation and reducing cholesterol. Those who are at risk or have suffered from a thrombotic episode are often put onto antiplatelet medication such as aspirin or clopidogrel. Each antiplatelet drug has a different target within the platelet, and is categorised by their mechanism of action. Approximately 12% of the UK population are currently taking the cholesterol-lowering drugs, statins. Taking AGE as a regular supplement could potentially provide the same benefits as that of antiplatelet or cholesterol-lowering drugs, whilst removing the side effects that are associated with taking any long-term medication.

Platelets profoundly contribute to the long-term progression of vascular diseases. Increased platelet reactivity can be a principal marker of subsequent cardiac events in those with CVD (5). The spontaneous aggregation of platelets can lead to the formation of clots and plaques increasing the risk of a thrombotic event. As AGE has been shown to inhibit platelet aggregation, it is therefore possible that AGE could help to prevent the formation of a plaque or thrombus or could even reduce the size of a thrombus already present (8) (Fig.1).

AGE could also benefit those with diabetes. Diabetics are at a much higher risk of a thrombotic event with mortality rates from CVD and stroke 3-5 times higher for patients with diabetes to those without. Diabetic platelets are hyper-reactive compared to non-diabetic platelets (9), and therefore have an even more crucial role in the progression to a thrombotic event.

Conclusion

AGE is beneficial in both the treatment and prevention of CVD by reducing many of the contributing factors in CVD. Studies indicate that AGE can inhibit platelet aggregation through a multi-mechanistic effect upon platelet signalling. Platelets have a crucial role in the progression of CVD, therefore those who are at risk of developing CVD or a thrombotic event may benefit from the regular consumption of AGE as a dietary supplement.

Acknowledgements

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