THE INHIBITORY MECHANISMS OF AGED GARLIC EXTRACT ON PLATELET AGGREGATION

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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 of the LJMU or other academic or professional institution.

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ABSTRACT

Cardiovascular disease is associated with multiple factors including the increased ability of platelets to aggregate. Natural compounds such as garlic are known to reduce the clinical onset of cardiovascular disease. Garlic is available in many forms commercially. One such form is aged garlic extract (AGE). It has been established through *in vitro* and *in vivo* testing that AGE can reduce the incidence of platelet aggregation stimulated by a variety of agonists; however, the mechanisms responsible for this observed inhibition have yet to be determined scientifically. Hence, the aim of this study was to examine and elucidate the inhibitory mechanisms of AGE on platelet aggregation both *in vitro* and *in vivo*.

In vitro testing using platelet aggregometry showed that platelets when incubated with AGE (1.56-25 %, v/v) showed a significant concentration-dependent reduction in platelet aggregation when challenged with the following agonists: adenosine diphosphate, arachidonic acid, A23187, adrenaline, collagen and thrombin. Individual constituents found in AGE were tested individually and as part of a mixture to observe any potential inhibitory effects. It was found that the constituents were not as efficient as AGE in its natural form in inhibiting platelet aggregation. Thus, all the constituents that make up AGE are needed in order to bring about an inhibition in platelet aggregation. The effects of AGE were compared to several known classical inhibitors of platelet aggregation induced by ADP; in order to establish the areas within the biochemical signalling pathway(s) where AGE may exert its inhibitory effect. From these experiments the following biochemical signalling pathways were identified as possible targets for AGE: arachidonic acid metabolism, calcium mobilisation, fibrinogen binding and cAMP levels.

Possible inhibitory mechanisms for AGE is via calcium mobilisation, in that the intracellular levels of calcium were significantly suppressed in the presence of AGE, when platelets were challenged with the agonists ADP and A23187. This was investigated using fluorescence spectrophotometry. Another area where AGE is exerting its inhibitory effect is via the final step in platelet aggregation (fibrinogen binding to its receptor GPIIb/IIIa) and an increase in intraplatelet levels of cAMP. AGE was found to significantly reduce platelet aggregate formation via fibrinogen binding. This was monitored using a simple adhesion assay and flow cytometry. AGE was also found to increase intracellular levels of cAMP when compared to the known inhibitor PGE_1. AGE had no effect upon arachidonic acid metabolism during platelet aggregation stimulated with the agonists ADP and thrombin.

In vivo testing where participants consumed 5 mL AGE/day for 14 days, showed that AGE significantly inhibited agonist-induced platelet aggregation. Of the biochemical parameters that were analysed it was found that AGE significantly increased the intraplatelet levels of cAMP when compared to the baseline (Day 0) and washout (Day 28) samples in both resting and platelets activated with ADP.

Therefore, it can be concluded that the inhibitory mechanisms of AGE with regards to platelet aggregation is via calcium mobilisation, fibrinogen binding, and increased cAMP *in vitro.* Whereas in *in vivo* testing, increased cAMP levels are the only biochemical signalling parameter that AGE has any inhibitory effect upon.

TABLE OF CONTENTS

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

5HT	Serotonin
AA	Arachidonic Acid
AC	Adenylate cyclase
ADP	Adenosine 5'diphosphate
AGE	Aged Garlic Extract
AI_3	Aluminium fluoride
AMP	Adenosine monophosphate
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BC	β-chlorogenin
BIS	Bisindolymaleimide
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
Ca^{2+}	Calcium ion
cGMP	Cyclic Guanosine Monophosphate
COX	Cyclooxygenase
CVD	Cardiovascular disease
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
EDTA	Ethyleledиamineetetacetic acid
EGTA	Ethylene glycol-bis-(2-amino-ethylether)-N,N,N',N'-tetra-acetic acid
EIA	Enzyme immunosorbent assay
FA	Fructosylarginine
FRET	Fluorescence Resonance Energy Transfer
Fura-2/AM	Fura-2 pentakis (acetylmethyl) ester
GEN	Genistein
G proteins	Glycol-proteins

GPIIb/IIIa	Glycoprotein IIa/IIIb
GTP	Guanosine triphosphate
HEPES	Hexadimethyldisilazane
IBMX	3-isobutyl-1-methylxanthine
IP_3	Phosphoinositol triphosphate
NAC	N-acetyl-L-cysteine
NaF	Sodium fluoride
NO	Nitric oxide
OKA	Okadaic acid
$P2Y_1$	ADP receptor
$P2Y_{12}$	ADP receptor
PA	Phosphatidic acid
PAF	Platelet-activating factor
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PG	Prostaglandin
PGE_1	Prostaglandin E_1
PGI_2	Prostacyclin
PIP_2	Phosphatidyl inositol bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol 12-myristate acetate
PMSF	Phenylmethylfulfonyl fluoride
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
SAC	S-allylcysteine
SAMC	S-allylmercaptocysteine
SEC	S-ethylcysteine
SMC	S-methylcysteine
SPC	S-propenylcysteine
TCA	Trichloroacetic acid

TLC	Thin-layer chromatography
TXA_2	Thromboxane A_2
vWf	von Willebrand factor

PUBLICATIONS ARISING FROM WORK

Allison, GL., Lowe, GM. And Rahman, K. (2006) Aged garlic extract and its constituents inhibit platelet aggregation through multiple mechanisms. *Journal of Nutrition*, 136 Suppl 3:S782-S788. (Appendix VIII)

Allison, GL, Lowe, GM. And Rahman, K. (2006) Aged garlic extract may inhibit aggregation in human platelets by suppressing calcium mobilisation. *Journal of Nutrition*, 136 Suppl 3: S789-S792. (Appendix VIII)

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DEDICATION

This thesis is dedicated in memoriam, with love to a wonderful friend and sister.

Miss Frances Dorothea Allison

(1981-2002)

"Sisters First, Friends Forever"

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. CARDIOVASCULAR DISEASE AND PLATELETS

In the United Kingdom and Western World, cardiovascular disease (CVD) including atherosclerosis, heart attack, stroke and heart failure is the leading cause of morbidity and mortality, with the death rates increasing steadily each year (Chambers 2004; Harrison 2005; Scott 2004; Willoughby *et al* 2002). Statistics gathered by the American Heart Association (americanheart.org) revealed that twice the number of people diagnosed with CVD die, than those suffering from all types of cancers combined. There are many risk factors associated with CVD some of which are not modifiable such as age, ethnicity, male gender and genetics (Scott 2004). There are other risk factors which can be modified by changes to lifestyle; these include diet, obesity, smoking and lack of exercise. CVD is not restricted to diseases of the heart as, imbalances in food intake, energy metabolism, blood pressure, inflammation (Vlasuk and Scarborough 2005) and increases in the prevalence of disease states such as obesity and diabetes, have significantly contributed to the overall increase in the occurrence of CVD. The disease process is a complex one in that CVD, especially atherosclerosis does not occur spontaneously. McBane *et al.* (2003) reported that autopsy studies have shown that atherosclerosis (general term for the thickening and hardening of arteries) begins during adolescence and continues to develop slowly over many years reaching clinical onset with the presentation of symptoms usually in middle age.

It has been suggested that platelets are involved in the development of atherosclerosis and the clinical onset of CVD, this is due to an increase in the number of aggregating platelets within the circulatory system (Banerjee and Maulik 2002; Gawaz 2006; Huo and Ley 2004; Imano *et al.* 2002; Keating *et al.* 2004; McBane *et al.* 2003; Rauch *et al.* 2001; Tan *et al.* 2004; VanWijk *et al.* 2003). In disease states such as atherosclerotic arteries, platelet aggregation plays a central role in coronary thrombosis and is related to a cascade of events including expression of adhesion molecules on the surface of the endothelium, the oxidation of lipoproteins, monocyte invasion of the vessel wall, foam cell formation, smooth muscle phenotypic change and proliferation and platelet deposition leading to myocardial infarction, stroke or sudden death (Andre *et al.* 2003; Dopheide *et al.* 2001; Huo and Ley 2004; Rahman 2001).

Historically, there have been 3 main theories of the development of atherosclerosis (Brydon *et al.* 2006; Gawaz 2006):

1. Incrustation theory – one of the earliest theories of atherosclerosis proposed by Rokitansky in 1852. He suggested that disease development was due to fibrin deposition on the arterial wall.
2. Lipid infiltration theory – involvement of plasma lipids and their cellular metabolism proposed by Virchow in 1858.
3. Irritation theory – chronic inflammation reaction proposed by Ross and Glomset in 1973.

In recent years atherosclerosis has been termed as a chronic inflammatory process in which leukocytes interact with the structurally intact but, dysfunctional endothelium of the arteries (Brydon *et al.* 2006). Platelets have been reported to be involved with the progression of this disease. However, their role in disease progression was always reported as occurring during the latter stages of atherosclerosis. Research conducted by Massberg *et al.* (2002) with Apo-E-deficient mice has shown that platelets play an important role in all stages of plaque development within arteries. Apo-E-deficient mice have elevated cholesterol levels, a common risk factor associated with atherosclerosis, and the mice also display widespread lesions that are similar in pathomorphology to humans with the disease. Fluorescence microscopy conducted *in vivo* showed that platelets were the first blood cell to arrive at the site of endothelial dysfunction, and that they adhered to the endothelium. Other studies have shown that platelet adhesion plays a critical role in the initiation and progression of atherosclerosis through leukocyte recruitment to the lesion site (Huo and Ley 2004; Massberg *et al.* 2002; Merten and Thiagarajan 2004). Adhesion of platelets to the endothelium triggers platelet activation and the rapid expression of adhesion molecules such as Platelet-selectin (P-selectin). P-selectin binds to the leukocyte receptor and promotes leukocyte recruitment to the endothelium. Activated platelets release a wide array of adhesive and pro-inflammatory factors, which are deposited on the surface of leukocytes and endothelial cells through P-selectin-mediated reactions. These include cytokines and chemokines which are powerful chemoattractant agents that stimulate leukocyte migration to the endothelium. All these events are responsible to some degree for the development of atherosclerosis. Human studies involving the observation of activated platelets via flow cytometry

suggest that the same processes are likely to occur in patients with atherosclerosis as reported by Brydon *et al.* (2006).

Platelets play an important role within the cardiovascular system (CVS), in that they are responsible for primary haemostatic functions through adhesion, aggregation and subsequent thrombus formation induced by agonists such as collagen, thrombin and many other factors exposed at sites of vascular injury (Andre *et al.* 2003; Payrastre *et al.* 2000). Thus, the inhibition of platelet activation and aggregation is important in maintaining constant blood flow throughout the CVS. In normal vasculature, circulating platelets interact passively with the endothelial lining of the vessel wall, due in part to the secretion of platelet-inhibiting substances such as prostacyclin and nitric oxide (Dopheide *et al.* 2001). It is upon vascular injury and the exposure of the sub-endothelial matrix that platelets become activated, aggregate and form a thrombus (platelet plug) to arrest the flow of blood from the damaged vessel site (Gregg and Goldschmidt-Clermont 2003; Keating *et al.* 2004; Puri 1999; Rauch *et al.* 2001; Willoughby *et al.* 2002). During CVD blood vessels that have been injured and modified by smoking, cholesterol, or high blood pressure develop fatty plaques that line the vessel wall; these plaques can rupture and cause platelets to form thrombi. Although no bleeding has occurred within the vessel the platelets are fooled into thinking that an injury has taken place that will cause loss of blood. Thus, a thrombus is formed within the blood vessel that restricts blood flow, leading to heart muscle death, heart attack and eventually death.

1.2. PLATELETS

Platelets were first described by Donné in 1842 and classical descriptions of the cells were further elaborated by Bizzozero in 1881, which recognised the importance of platelet clumping in the blood clotting process (Freedman 2003; Dopheide *et al.* 2001). Platelets are non-nucleated cells that circulate freely in the blood as discoid shaped cells in a resting state. They are formed in the bone marrow via a process known as thrombocytopoesis from megakaryotic cells, which differentiate from pluripotent stem cells by a complex process that is orchestrated by a series of growth factors (Battinelli *et al.* 2001; Kaushansky 1996; Kaushansky 1997), and have a life span of approximately 8-12 days (Harrison 2005; Jurk and Kehrel 2005; Solet *et al.* 2001; Willoughby *et al.* 2002;). Platelets are the smallest and second most numerous blood cells in the circulatory system with dimensions of approximately 1.0-2.0 by 0.5 μM. They are present in the circulation at a volume of 150-450 x 10^9 platelets/L (Dopheide *et al.* 2001; Harrison 2005).

Platelets have a general cell physiology (Figure 1.1) in that they contain a plasma membrane, internal membrane (open canalicular and dense tubular systems), a cytoskeleton (microtubules and microfilaments), mitochondria, glycogen granules, storage granules (α-granules and dense granules), lysosomes (acid proteases, acid glycosidases, acid phosphatases, acid sulphates) and peroxisomes (catalase). The α-granules are important to platelet function in that they store and secrete proteins, such as fibrinogen, von Willebrand factor (vWf), platelet factor IV β-thromboglobulin and platelet-derived growth factor. Dense granules store smaller molecules (serotonin, ATP, ADP, Ca^{2+}, pyrophosphate) and are responsible for sustaining platelet aggregation, which is necessary for the formation of a haemostatic plug. Platelet membranes contain a vast array of receptors and receptor-like proteins that are responsible for cell-to-cell adhesion and the biochemical signalling pathways responsible for platelet aggregation. These receptors also allow for the adherence of platelets to exposed sub-endothelial matrix at sites of vessel injury.

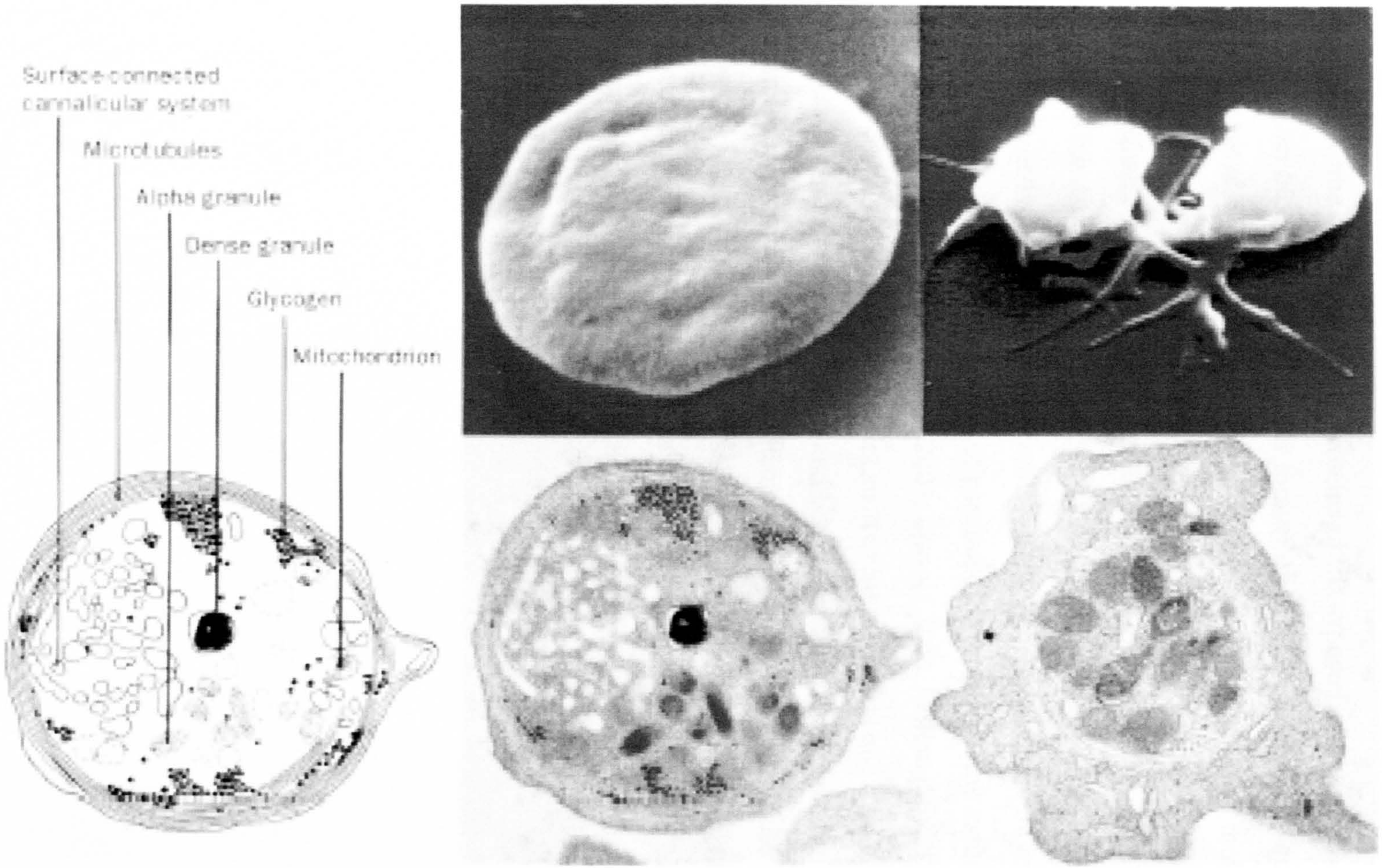


Figure 1.1. Schematic representation and electron micrograph of a resting and activated platelet. Top right photographs show resting circulating platelets and bottom right pictures are of activated platelets. Taken from George (2000)

1.2.1 Platelet activation and aggregation

Platelet activation occurs in two steps, activation and aggregation (Figure 1.2) and is a complex biochemical process that requires the co-activation of many signalling pathways within and outside the platelet. In order for platelets to become activated they first need to come into contact with proteins of the exposed endothelial matrix as a result of either blood vessel damage or rupture of an atherosclerotic plaque (Chambers 2004; Solet *et al.* 2001). Collagen ,vWf, and fibronectin are adhesive molecules found within the sub-endothelial matrix that when bound to their respective receptor on the platelet membrane results in rapid platelet activation (Jackson *et al.* 2003). Upon adhesion to the damaged blood vessel platelets change shape from an inactivated discoid to an activated 'spiny' shape through contractile rearrangement of the cytoskeleton leading to the formation of pseudopods (long dendritic extensions) facilitating adhesion (Dopheide *et al.* 2001; George 2000; Willoughby 2002). Organelles contained within the platelet such as mitochondria and storage granules are contracted towards the platelet centre and become enclosed in a tight-fitting ring of reassembled microtubules and microfilaments (Willoughby 2002; White 1999). The last stage in activation is the expulsion of secretory organelle contents. Activated platelets form stable adhesion contacts with the damaged vessel wall and spread out over the injured surface to form a pseudoendothelium.

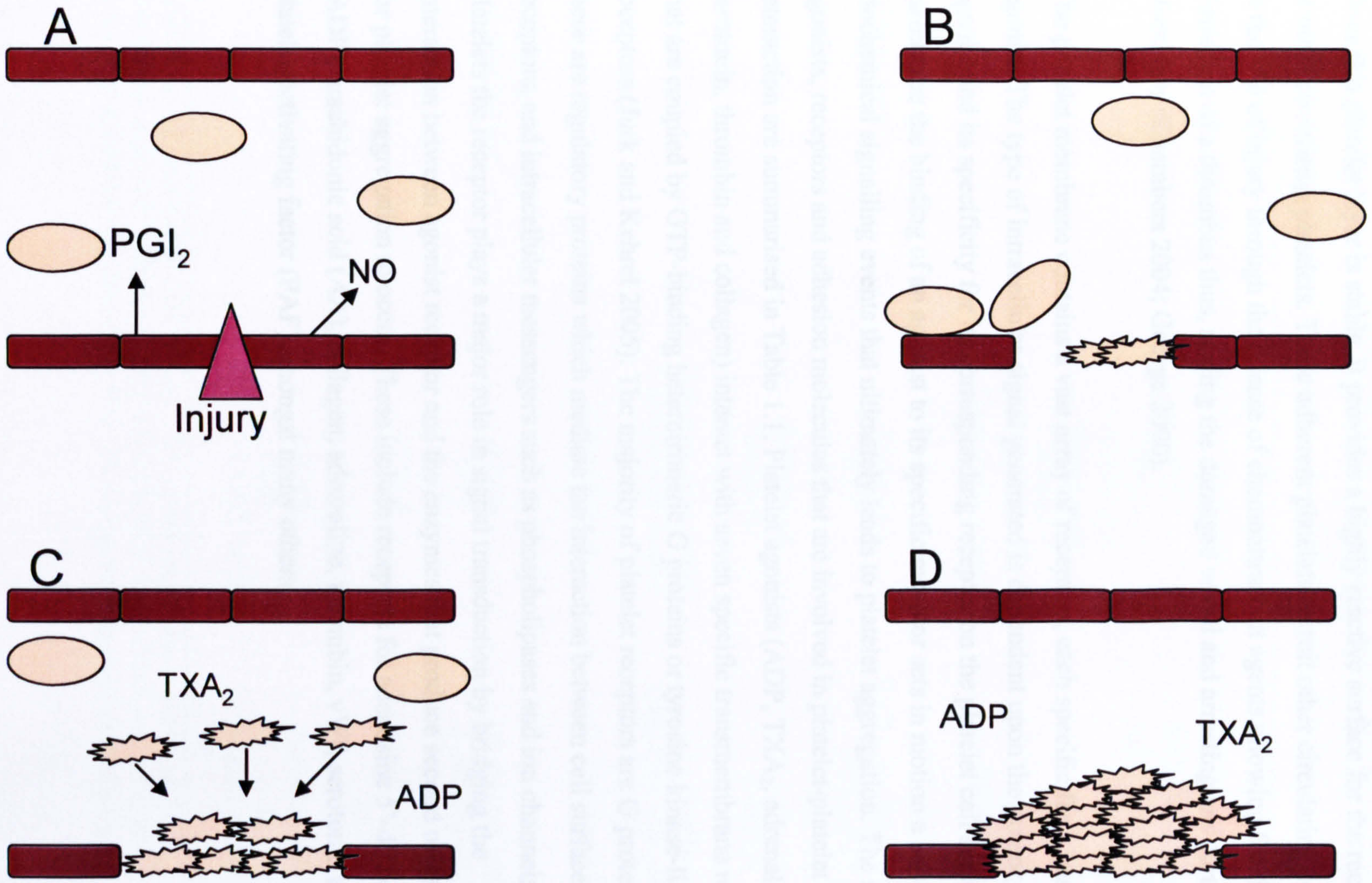


Figure 1.2. A schematic representation of the steps involved in platelet activation and aggregation. (A) Injury occurs to the blood vessel endothelium. (B) The subendothelial matrix proteins (vWf, collagen, fibronectin) are exposed and circulating resting platelets are attracted to the injury site where they become activated. (C) A platelet monolayer coats the injury site and more circulating platelets are recruited to the site via the release of platelet activating agonists. (D) Thrombus formation. Key: ADP, adenosine diphosphate; NO, nitric oxide; PGI_2, prostacyclin; TXA_2, thromboxane A_2.

Once this platelet layer is stable, it provides a highly reactive surface for the recruitment of other circulating platelets. These adherent platelets recruit other circulating platelets to the site of injury through the release of chemoattractant agents allowing for the formation of a thrombus thus, sealing the damaged vessel and arresting any further blood loss (Chambers 2004; George 2000).

The platelet membrane contains a vast array of receptors, each specific for a particular agonist. The type of intracellular signal generated is dependent upon the potency of the agonist and its specificity for its corresponding receptor on the platelet cell surface membrane the binding of an agonist to its specific receptor sets in motion a series of biochemical signalling events that ultimately leads to platelet aggregation. The major agonists, receptors and adhesion molecules that are involved in platelet-platelet interaction are summarised in Table 1.1. Platelet agonists (ADP, TXA_2, adrenaline, serotonin, thrombin and collagen) interact with seven specific transmembrane receptors that are coupled by GTP-binding heterotrimeric G proteins or tyrosine kinase-linked receptors (Jurk and Kehrel 2005). The majority of platelet receptors are G proteins and these are regulatory proteins which mediate the interaction between cell surface receptors, and intracellular messengers such as phospholipases and ion channels. In platelets the receptor plays a major role in signal transduction by bridging the interaction between agonist receptor and the enzymes that produce second messengers for platelet aggregation to occur. These include receptors for adenosine 5'-diphosphate (ADP), arachidonic acid (AA), collagen, adrenaline, thrombin, vWf, serotonin and platelet-activating factor (PAF) amongst many others.

Table 1.1. Agonists, ligands and receptors important for platelet function

Platelet Function	Agonists, ligands	Receptors
Initial and firm adhesion	vWf	GPIb/V/IX
	TSP1	GPIb/V/IX, CD36
	Collagen	$\alpha_2\beta_1$, GPVI, CD36
	Fibrinogen	$\alpha IIb\beta_3$
	Fibronectin	$\alpha_5\beta_1$
	Vitronectin	$\alpha_V\beta_3$
	Leminin	$\alpha_6\beta_1$
	High shear stress	GPIb/V/IX
Activation and amplification	Thrombin	PAR1, PAR4, GPIb/V/IX
	ADP	$P2Y_1$, $P2Y_{12}$
	TXA_2	TPα, TPβ
	Adrenaline	α_{2A}
	Serotonin	5-HT2A
	MMP-2, MMP-1	?
	Immune complexes	FcγIIa
	Complement factors	C1q, C3a, C5a receptors
	Plasmin	? unknown
	Streptokinase	? unknown
Aggregation/ amplification and stabilisation	Fibrin	Activated $\alpha IIb\beta_3$
	vWf	Activated $\alpha IIb\beta_3$, GPIb/V/IX
	TSP-1	Activated $\alpha IIb\beta_3$, CD36, IAP
	Fibronectin	Activated $\alpha IIb\beta_3$
	sCD40L	Activated $\alpha IIb\beta_3$
	Gas6	Axl
	SDF-1, TARC, MDC	CXCR4, CCRΛ

Taken from Jurk and Kehrel (2005). Key: TSP1, thrombospondin-1; MMP, matrix metalloproteinase; IAP, integrin associated protein; SDF, stromal cell-derived factor; TARC, thymus and activation-regulated chemokine; MDC, macrophage-derived chemokine.

In general, following agonist interaction with its receptor an inhibitory signal is sent to adenylate cyclase (AC). This enzyme is involved in the formation of cyclic adenosine monophosphate (cAMP) – an inhibitor of calcium mobilisation within the cell thus inhibiting platelet aggregation. Therefore, it is essential that the production of cAMP is discontinued allowing for aggregation to occur. The regulation of cAMP is an important mechanism for the suppression of platelet activation and aggregation. A rise in intraplatelet cAMP via AC or through the inhibition of phosphodiesterase leads to the sequestration of calcium within the cell. cAMP levels are increased through the stimulatory actions of the prostaglandins: prostacyclin (PGI_2) and prostaglandin E_1 (PGE_1). Another signal is relayed to the plasma membrane where AA is metabolised from phospholipids by activated phospholipase A_2 (PLA_2). The AA can be converted into prostaglandin endoperoxides (PGG_2/PGH_2) by cyclooxgenase (COX) and subsequently to thromboxane A_2 (TXA_2) by thromboxane synthase (TS), leading to the mobilisation of calcium from intracellular stores within the platelet to aid in cytoskeletal reorganisation and the formation of pseudopodia. By-products of COX and TS reactions diffuse out of the platelet membrane and bind to surface receptors leading to the activation of phospholipase C (PLC).

PLC catalyses the hydrolysis of phosphatidyl inositol bisphosphate (PIP_2) into the second messengers: phosphoinositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates an influx of calcium from internal stores in the endoplasmic reticulum; the calcium along with DAG activates protein kinase C (PKC). Activation of PKC leads to rearrangement of the cytoskeleton and subsequent platelet shape change, during which the contents of the dense granules (α, δ and λ) are released into the surrounding extracellular environment. Substances such as; ADP and serotonin (5HT) aid in the amplification of the platelet response and recruitment of nearby platelets which join the platelet plug. The agonist also induces the exposure of the conformationally competent GP IIb-IIIa complex (fibrinogen receptor) via an inside-out signalling mechanism which, in the presence of released calcium ions (Ca^{2+}) binds to fibrinogen which allows for the cross-linking of platelets thus facilitating platelet aggregation and the formation of a thrombus (platelet aggregate) (Andre *et al.* 2003; Dopheide *et al.* 2001; Solet *et al.* 2001). The cell signalling events involved in ADP platelet activation/ aggregation are summarised in Figure 1.3.

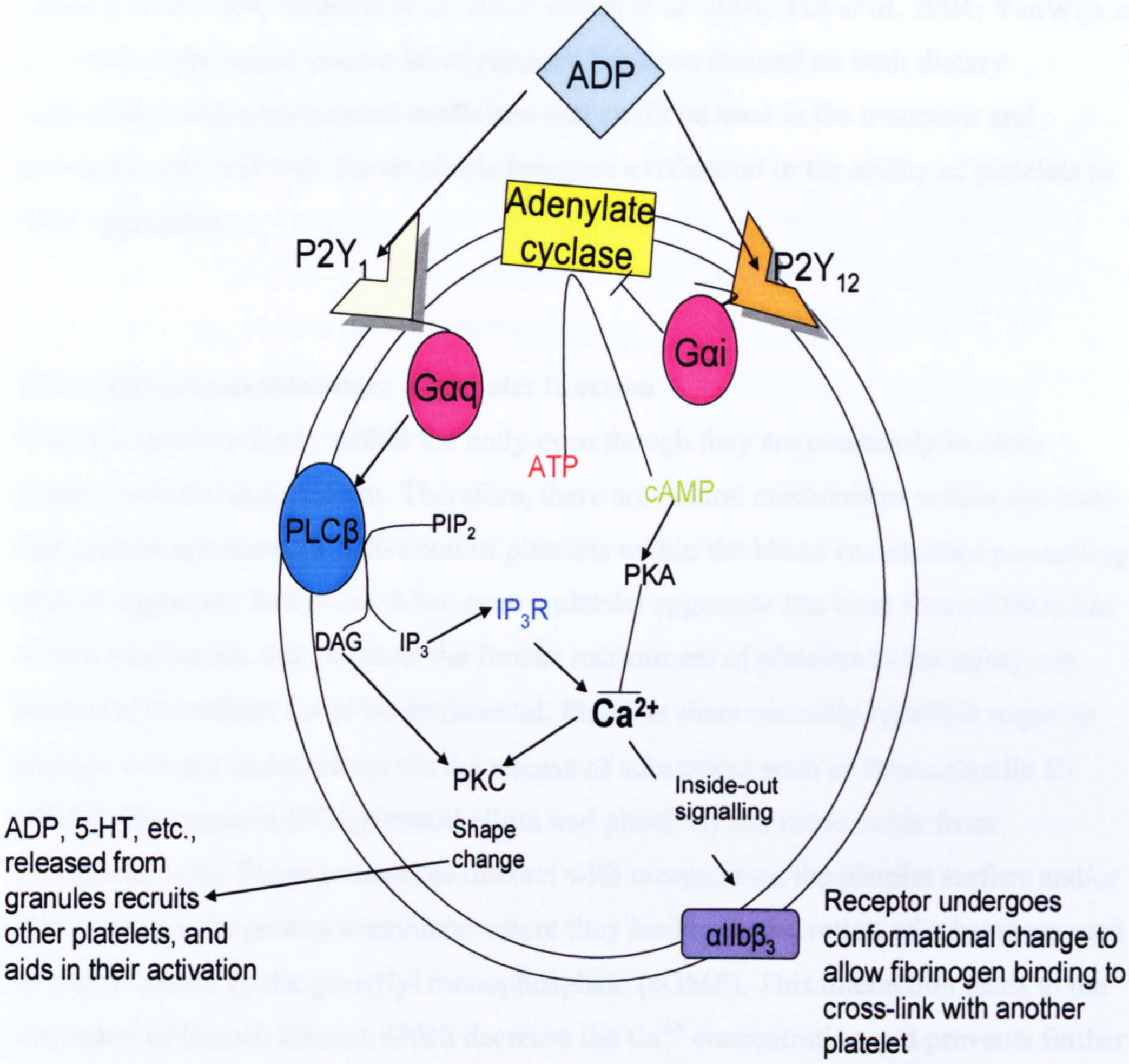


Figure 1.3. The biochemical events involved in ADP-induced platelet activation. ADP binds to its respective receptors $P2Y_1$ and $P2Y_{12}$ on the platelet cell surface. Simultaneous binding of both receptors is required for platelet aggregation. ADP-receptor interaction triggers a series of events that ultimately leads to platelet aggregation. The $P2Y_1$ receptor stimulates the hydrolysis of PIP_2 into IP_3 and DAG via the enzyme PLCβ. IP_3 binds to its receptor IP_3R on the endoplasmic reticulum to release calcium ions into the intracellular environment. Ca^{2+} and DAG activate PKC to rearrange the cytoskeleton and the release of dense granule contents into the extracellular environment. These include substances such as ADP, fibrinogen, 5-HT etc. which aid in the recruitment and activation of nearby platelets. Ca^{2+} and other inside-out signalling mechanisms allow for the fibrinogen receptor $\alpha IIb\beta_3$ to undergo a conformational change allowing for the binding of fibrinogen to its receptor. The ADP receptor $P2Y_{12}$ inhibits the actions of the enzyme adenylate cyclase and suppresses the levels of intracellular cAMP. Key: PIP_2-phosphatidyl inositol bisphosphate; IP_3-phosphoinositol triphosphate; DAG-diacylglycerol; PLCß-phospholipase C beta; IP_3R-phosphoinositol triphosphate receptor; PKC-protein kinase C; 5-HT-serotonin.

1.3. INHIBITORS OF PLATELET AGGREGATION FOR THE TREATMENT AND PREVENTION OF CARDIOVASCULAR DISEASE

Platelet aggregation plays a significant role in the development and progression of CVD (Banerjee and Maulik 2002; Gawaz 2006; Huo and Ley 2004; Imano *et al.* 2002; Keating *et al.* 2004; McBane *et al.* 2003; Rauch *et al.* 2001; Tan *et al.* 2004; VanWijk *et al.* 2003). Over recent years a lot of research has been focused on both dietary supplements and conventional medicines that could be used in the treatment and prevention of CVD with the emphasis being on a reduction in the ability of platelets to form aggregates.

1.3.1 Endogenous inhibitors of platelet function

Platelets circulate freely within the body even though they are constantly in close contact with the endothelium. Therefore, there are natural mechanisms within the body that prevent spontaneous activation of platelets within the blood vessels thus preventing platelet aggregate formation. Also, once a platelet aggregate has been formed there has to be a mechanism that prevents the further recruitment of platelets to the injury site otherwise the effects could be detrimental. Platelets share mutually repellent negative charges with the endothelium via the release of substances such as Prostaglandin E_1 (PGE_1), Prostacyclin (PGI_2) (endothelium and platelets) and nitric oxide from endothelial cells. These substances interact with receptors on the platelet surface and/or diffuse across the plasma membrane where they lead to the secretion of substances such as cAMP and/or cyclic guanylyl monophosphate (cGMP). This interaction leads to the activation of protein kinases which decrease the Ca^{2+} concentration and prevents further platelet activation and aggregation from occurring. Another process by which the body controls platelet activation and aggregation is through the breakdown of extracellular ADP to AMP by the enzyme ecto-ATPdase (adenosine triphosphate disphosphorylase), which is expressed by endothelial cells (Tan *et al.* 2004).

1.3.2 Pharmacological inhibition of platelet function

In disease states the regulatory mechanisms that occur in healthy individuals cannot be relied upon to function normally to suppress the development of disease. Hence, pharmacological intervention is used in an attempt to redress the balance and hopefully halt further disease progression and return the body to normal physiological conditions.

The medicines currently available on the market can be divided into 3 groups: cyclooxygenase (COX) inhibitors, ADP receptor antagonists and GPIIb/IIIa receptor antagonists.

1.3.2.1. Cyclooxygenase inhibitors in the control of platelet function

The most established COX inhibitor to-date is aspirin. It is an effective anti-platelet drug which has multiple dose-dependent therapeutic effects (Clutton *et al.* 2001). Aspirin exerts its inhibitory effect upon platelet activation by irreversibly suppressing thromboxane A_2 synthesis during arachidonate metabolism. This is achieved by rendering the enzyme COX inactive through acetylation, without this enzyme arachidonate metabolism cannot continue and the platelet aggregatory process is stopped. It has been found clinically that administration of aspirin produces dose-dependent inhibition of platelet COX activity after a single oral dose. The inhibitory effect of aspirin can be observed as quickly as 5 min after oral administration, and the inhibitory effect is maintained for the entire life-span of the platelet. This is because platelets lack the synthetic machinery to generate significant amounts of new COX (Michelson 2004), whose activity only returns when new platelets are generated and released from the bone marrow into the circulatory system.

1.3.2.2. ADP receptor antagonists and their effect on the control of platelet function

ADP plays an important role in platelet activation and subsequent aggregation. Upon its release from dense granules it binds to its receptors P2Y1 and P2Y12 (Hardy *et al.* 2005), to initiate calcium mobilisation, and inhibit adenylyl cyclase activity, respectively (Gachet 2001; Puri 1999). For platelet activation and aggregation to occur successfully, it is essential that both ADP receptors are occupied with ADP. Thus, if one of the receptors were to be antagonised platelet aggregation would not be allowed to continue. There are two ADP receptor antagonists available clinically these being clopidogrel and ticlopidine.

1.3.2.3. GPIIb-IIIa receptor antagonists

GPIIb-IIIa receptor antagonism has a strong anti-platelet effect; this inhibition is due to interference of the final common pathway of platelet aggregation and is not dependent on a single activation pathway. To-date there are 3 main GPIIb-IIIa antagonists clinically available for the treatment of cardiovascular disease these are: abciximab (a

chimeric monoclonal antibody), eptifibatide (a cyclic heptapeptide) and tirofiban (a nonpeptide tyrosine derivative), all of which are administered intravenously for maximum effect.

1.3.3. Natural (alternative) inhibition of platelet function

Pharmacological intervention can produce promising results, however it is not without risk. As prolonged treatment with drugs such as aspirin can result in the treatment no longer being effective and the patients can often develop a resistance to the drug rendering it ineffective. Therefore, both researchers and clinicians are always looking for alternative preventative and treatment methods. Research over the past 40 years has shown that the use of dietary supplements such as green tea extract, ginger, kinetin, glucosamine, have caused a significant reduction in platelet aggregation both *in vivo* and *in vitro* studies (Hua *et al.* 2004; Nurtjahja-Tjendraputra *et al.* 2003; Sheu *et al.* 2003; Son *et al.* 2004). Not only dietary supplementation but, foods in general can also inhibit platelet aggregation such as tomatoes, onions, chives, kiwi fruit, dark chocolate, turmeric, red wine (polyphenols present in wine) (de Lange *et al.* 2003; Duttaroy *et al.* 2002; Innes *et al.* 2003; Moon *et al.* 2000; Osmont *et al.* 2003; Shah *et al.* 1999). One such food that has been researched extensively for its cardioprotective properties including its inhibition of platelet aggregation is garlic (Table 1.2).

There are many benefits into researching alternative therapies for CVD. The primary reason is that the herb of interest is already well-established as a treatment/preventative method through its use in folklore medicine. Studies indicate that these herbs have multifactorial effects within the body. It is often expensive and time-consuming to research conventional treatments, as there are a number of stages of development and testing that needs to be done before it can be given to humans in clinical trials. Herbs have a variety of components that target many different cellular processes simultaneously whereas pharmacological trials look at the effects of one particular active constituent. Many pharmacological studies do not reach completion in that the constituent under investigation proves effective during initial testing but, often fails during clinical trials due to in part to the side effects that are associated with its use. New regulations and legislations into the therapeutic use of herbal remedies have been introduced in Europe by the European Union. This has caused an increase in the number

of scientific studies researching the protective and preventive properties of herbs to ensure that they actually are beneficial in the treatment of diseases such as CVD (Kroes 2006).

Herbal use throughout the world has reached high prevalence over the past 20 years Further research is necessary to elucidate the pharmacological activities of many herbal therapies now being used to treat CVD. This is the primary reason why it is important to conduct studies such as this one.

Table 1.2. Garlic as an inhibitor of platelet aggregation: *in vitro* and *in vivo* studies

Ref	Preparation	Study Type	Effect
Bordia *et al.* (1978)	Essential oil of garlic	*In vitro*	inhibition
Makheja *et al.* (1980)	Garlic extract	*In vivo*	inhibition
Vanderhoek *et al.* (1980)	Garlic oil	*In vitro*	inhibition
Makheja *et al.* (1980)	Garlic extract	*In vitro*	inhibition
Ariga *et al.* (1981)	Essential oil, methyl-allyl-trisulphide (MATS)	*In vitro*	Dose-dependent inhibition
Boullin (1981)	Fresh garlic	*In vivo*, 4h	inhibition
Bordia *et al.* (1982)	Garlic oil	Double-blind	inhibition
Apitz-Castro *et al.* (1983)	Methanol extract of garlic	*In vitro*	inhibition
Barrie *et al.* (1987)	Garlic oil, diallylsulphide, diallyltrisulphide (DADS, DATS)	*In vivo*, 3 months	Inhibition decreased with garlic administration
Harenburg *et al.* (1988)	600 mg, dried garlic	*In vivo*	No inhibition
Kiesewetter *et al.* (1991)	Garlic powder	*In vivo*, 4 weeks	inhibition
Legnani *et al.* (1993)	Chinese garlic powder	*In vivo*, 14 days	inhibition
Steiner *et al.* (1994)	Aged Garlic Extract (AGE)	*in vivo*, 6 months	Inhibition
Morris *et al.* (1995)	Oil extract of garlic	*In vivo/ in vitro*	Inhibition *in vitro*/ no *inhibition in vivo*

Ali and Thompson (1995)	Fresh garlic	*In vitro/ in vivo*	inhibition
Agarwal *et al.* (1997)	cycloalliin	*In vivo*	inhibition
Steiner and Lin (1998)	AGE	*In vivo*, 11 months	inhibition
Qi *et al.* (2000)	Diallyl trisulfide	*In vitro*	inhibition
Rahman and Billington (2000)	AGE	*In vivo*, 13 weeks	inhibition
Steiner and Lin (2001)	AGE	*In vivo*, 6 weeks	inhibition
Chan *et al.* (2003)	Diallylsulfide, diallyl disulphide, diallyltrisulphide	*In vitro*	inhibition
Chang *et al.* (2004)	Alk(en)yl thiosulfate	*In vitro*	inhibition
Allison *et al.* (2006)	AGE, Individual AGE constituents	*In vitro*	Inhibition Dose-dependent No inhibition with individual constituents of AGE

1.4. GARLIC

Garlic (*Allium sativum*) derived from the Old English word *garleac,* meaning "spear leek" dates back over 6000 years from its origins in Central Asia. It is a member of the lily (*Liliaceae*) family which also includes: onions, leeks, chives and shallots. Garlic has become domesticated over the years to suit different climates with over 300 varieties of the herb grown world wide. One of the most common varieties of garlic is American garlic which has white papery skin and is strong in flavour, other types include Mexican garlic which is pink in colour and has a medium flavour. Throughout history there are many references made in folk medicine to the use of this plant as a treatment for a variety of ailments. These include in the UK as a cold remedy, in certain parts of Asia it has been used in the treatment of conditions such as 'whooping cough,' fungal and bacteria infections to name a few.

1.4.1 Garlic and cardiovascular disease – the benefits

Research conducted on the benefits of garlic consumption with regards to CVD has been carried out over the past 40 years and has shown that garlic has a positive effect on lowering its incidence. Studies have shown that garlic intake is associated with a lowering of blood glucose levels, a reduction in blood pressure, cholesterol levels, and it also inhibits platelet aggregation (Banerjee and Maulik 2002; Barrie *et al.* 1987; Berthold and Sudhop 1998; Bordia *et al.* 1998; Fugh-Berman 2000; Orekhov and Grunwald 1997; Rahman and Lowe 2006; Tattelman 2005)

1.4.2. Commercially available garlic products

As garlic is very prominent in history in the treatment of CVD, many scientists and dietary supplement manufacturers have looked at alternative ways of processing raw garlic for supplementation into the diet. These can be found today on the shelves of many health food stores, and include: garlic tablets, aged garlic extract, oil of steam-distilled garlic, oil of oil-macerated garlic, ether extracted oil of garlic, liquid garlic. Each is processed in different ways and contain markedly different sets of compounds thus, making studies of comparison extremely difficult for researchers (Table 1.3). The garlic dietary supplement of interest in this particular study is aged garlic extract (Kyolic).

Table 1.3. Commercially available garlic products, their main compounds and characteristics.

Product	Main compounds and characteristics
Garlic essential oil	1 % oil-soluble sulphur compounds (e.g., DAS or DADS) in 99 % vegetable oil
	No water-soluble fraction
	No allicin
	Not well standardised
	No safety data
Garlic oil macerate	Oil-soluble sulphur compounds and alliin
	No allicin
	Not well standardised
	No safety data
Garlic powder	Alliin and a small amount of oil-soluble sulphur compounds
	No allicin
	Not well standardised
	Results on cholesterol are not consistent
	No safety data
Aged garlic extract	Mainly water-soluble compounds (e.g. SAC, SAMC or saponins)
	Standardised with SAC
	Small amount of oil-soluble sulphur compounds
	Various beneficial effects
	Well-established safety
	Heavily researched (300+ papers)

Taken from Amagase *et al.* (2001)
Abbreviations: DAS, diallyl sulphide; DADS, diallyl disulphide; SAC, s-allyl cysteine; SAMC, s-allyl mercaptocysteine.

1.5. AGED GARLIC EXTRACT

AGE is produced by Wakunaga of America, Co., Ltd, Mission Viejo, USA. AGE is produced from organically grown garlic and is aged for up to 20 months in stainless steel tanks. This cold aging process modifies the raw garlic into a unique complex chemical mixture via natural chemical and enzymatic reactions. It is standardised with S-allyl-$_L$-cysteine (SAC), a safe and stable organosulphur compound derived from garlic as a means of quality control and lot-to-lot product uniformity. AGE contains no more than 0.05 % SAC by dry weight (1.47 g/l). Scientific testing has confirmed through toxicological analysis that AGE is safe for human consumption and has no adverse side effects associated with its prolonged use (Lawson and Gardner 2005; Hoshino *et al.* 2001; Kyolic 2006).

1.5.1. Chemical composition of AGE

Chemical constituents contained within raw garlic include: sulphur-containing compounds such as ajoene, alliin, cysteine sulfoxides, trisulfides etc, at least 17 amino acids, and a variety of vitamins and minerals (Brace 2002). The general composition of fresh garlic is shown in Table 1.4. The major component of garlic is water, other components present include: carbohydrates, sulphur compounds, protein, fibre, amino acids, saponins, vitamins and minerals (Lawson 1996). The characteristic flavour and aroma associated with garlic is due to the organosulphur compounds (Table 1.5) and the biological activity of this herb is attributed to these particular constituents (Agarwal 1996; Block 1985; Block 1992; Rose *et al.* 2005; Tapiero *et al.* 2004; Xiao and Parkin 2002). AGE differs in chemical composition to fresh garlic in that most of the components responsible for characteristics such as odour (thiosulfinates) are removed during the aging process. The major components found in AGE are water-soluble organosulphur compounds, and also present are unique biochemical constituents not found in fresh garlic: S-allyl-L-cysteine, fructosylarganine and 1,2,3,4-tetrahydro-ß-carboline-3-carboxylic acids (Ryu and Rosen 2003) but are formed and their content is increased during manufacture. During the aging process γ-glutamyl-S-allylcysteine, the parent compound to alliin is converted to S-allylcysteine (SAC), S-allylmercaptocysteine (SAMC) and others. Weinberg *et al.* (1992) developed a method which detected and identified nine oil-soluble organosulphur compounds in AGE, these include: diallyl sulphide; triallyl sulphide; diallyl disulphide and diallyl polysulphide.

Several organosulphur compounds have been identified and isolated from AGE. These will be discussed in more detail in chapter 3.

Table 1.4. General composition of garlic

Component	Amount (fresh weight; %)
Water	62-68
Carbohydrates	26-30
Protein	1.5-2.1
Amino acids: common	1-1.5
Amino acids: cysteine sulphoxides	0.6-1.9
γ-Glutamylcysteines	0.5-1.6
Lipids	0.1-0.2
Fibre	1.5
Total sulphur compounds	1.1-3.5
Sulphur	0.23-0.37
Nitrogen	0.6-1.3
Minerals	0.7
Vitamins	0.0015
Saponins	0.04-0.11
Total oil-soluble compounds	0.15
Total water-soluble compounds	97

Taken from Lawson (1996)

Table 1.5. Principal organosulphur compounds found in garlic

Compound	mg/g fresh weight
S-(+)-Alkyl-$_L$-cysteine sulphoxides	
Allylcysteine sulphoxide (alliin)	6-14
Methylcysteine sulphoxide (methiin)	0.5-2
Trans-1-Propenylcysteine sulphoxide (isoalliin)	0.1-1.2
Cycloalliin	0.5-1.5
γ-$_L$-Glutamyl-S-alkyl-$_L$-propenylcysteine	
γ-Glutamyl-S-*trans*-1-propenylcysteine	3-9
γ-Glutamyl-S-allylcysteine	2-6
γ-Glutamyl-S-methylcysteine	0.1-0.4

Taken from Lawson (1998)

1.5.2. Therapeutic actions of AGE

Since its development in 1955, there have been more than 350 scientific research studies conducted on the therapeutic actions of AGE and its constituents (Wakunaga of America; www.kyolic.com). The following therapeutic properties have been identified: cardioprotective, liver-protective, immune enhancement, antioxidant and radioprotective effects, anti-stress and anti-fatigue, anti-cancer, neurotrophic, anti-aging, anti-depression, anti-fungal, and anti-bacterial (Wakunaga of America, www.kyolic.com). The studies are a combination of both *in vitro* and *in vivo* testing. Clinical studies have shown that AGE is proficient in its ability to reduce cardiovascular risk factors associated with disease progression and these include: a reduction of blood lipids (serum cholesterol and triglycerides); inhibition of platelet aggregation; and a reduction in blood pressure following AGE administration for a specified period of time. Since this study is concerned with the effects of AGE on platelet aggregation, the cardioprotective properties of AGE found in clinical studies are summarised in Table 1.6 (a brief summary of the various clinical studies that looked at the cardioprotective actions of AGE).

Table 1.6. The cardioprotective effects of AGE – Clinical studies

Risk factor	Study
Lipid reduction	Lau *et al.* (1987) AGE was administered to subject's with↑ cholesterol for 6 months. Results: ↓ serum cholesterol, ↓ LDL cholesterol, ↑ HDL cholesterol. No change in the placebo group. Yeh *et al.* (1995,1997) 5 month double-blind, randomised, placebo controlled trial of men with ↑ cholesterol. Results: ↓ total plasma cholesterol, ↓ LDL cholesterol.
Inhibition of platelet aggregation	Rahman and Billington (2000) 13 week randomised double-blind study. ↓ platelet aggregation induced with ADP.
Lowering of blood pressure	Steiner and Lin (1996a) 6 month double-blind crossover study. ↓ systolic and ↓ diastolic blood pressure. Steiner and Lin (1996b) 6 month double-blind, placebo controlled, crossover study of hyperlipidemic patients. ↓ systolic blood pressure.
Inhibits plaque formation	Budoff *et al.* (2004) Randomised double-blind placebo controlled pilot study in heart surgery patients. ↓ inhibits plaque formation in the coronary artery.

1.6. AIMS AND OBJECTIVES OF STUDY

The first aim of this study was to investigate the effects of AGE and its constituents on agonist-induced platelet aggregation *in vitro*.

- The effect of AGE on platelet aggregation induced by a variety of agonists was investigated using platelet aggregometry.
- Individual components isolated from AGE and mixtures of these components were added to platelets and their inhibitory effect was monitored on platelet aggregation induced with the agonist ADP.
- Known classical inhibitors of platelet aggregation were compared to the inhibitory actions of AGE in platelets aggregated with ADP in order to elucidate a possible mechanism behind the observed inhibition of platelet aggregation with AGE.

The second aim of this study was to try and establish a possible inhibitory mechanism for AGE on ADP-induced platelet aggregation. This was achieved by breaking down the biochemical signalling pathways involved in platelet aggregation *in vitro*. The effects of AGE on the following pathways was monitored:

- The effects of AGE on calcium mobilisation were monitored using platelet aggregometry, fluorescent microscopy and fluorescent spectrophotometry.
- To investigate the effects of AGE on arachidonic acid (AA) metabolism when platelets were stimulated with the agonist ADP, was assessed using thin-layer chromatography (TLC).
- The effect of AGE on the ability of fibrinogen to bind to its receptor GPIIb-IIIa to produce a stable platelet aggregate *in vitro* was measured using four independent methods: disaggregation, a simple adhesion assay, flow cytometry and SEM imaging.
- cAMP levels and the potential effect AGE has upon this nucleotide was assessed using a competitive ELISA assay.
- To determine if AGE has any proteolytic activity was monitored by an azocasein assay and SDS-PAGE analysis, respectively.

The final aim of this study was to investigate and identify an inhibitory mechanism for AGE on platelet aggregation *in vivo.*

- The *ex vivo* effect of AGE on ADP-induced platelet aggregation was measured using platelet aggregometry. Whole blood was incubated with AGE, the platelets were removed and aggregation curves were analysed and compared to that of a control.
- Human clinical trial. Volunteers were recruited from the university population, and blood samples were taken at Day 0 (baseline), Day 14 (following AGE administration, 5 ml/day) and Day 28 (washout). The platelets were removed from the whole blood and the following parameters were tested: platelet aggregation induced by the agonists: ADP, AA, adrenaline and collagen, intracellular cAMP levels were monitored using an EIA assay, the expression and binding capabilities of the fibrinogen receptor GPIIb/IIIa (fibrinogen receptor expression on platelets were measured using flow cytometry), and total cholesterol was also determined.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Chemicals

Adenosine diphosphate (ADP), aspirin (acetylsalicyclic acid), A23187, AG527, aluminium fluoride (AlF_3), azocasein, L-arginine, L-cysteine, L-methionine, bisindolymaleimide (BIS), Bovine Serum Albumin (BSA), Bradford reagent, bromophenol blue, coomassie brilliant blue G-250, chymotrypsin, dimethyl sulfoxide (DMSO), ethylenediamineetetaacetic acid (EDTA), ethylene glycol-bis(2-amino-ethylether)-N,N,N',N'-tetra-acetic acid (EGTA), ethyl acetate, fibrinogen fraction I Type IV: from Bovine plasma, fixer and replenisher, Fura-2 pentakis (acetoxymethyl) ester (Fura-2/AM), genistein, glycerol, glycine, HEPES, hexadimethyldisilazane, 3-isobutyl-1-methylxanthine (IBMX), immunoglobulin G (IgG), indomethacin, 2-mercaptoethanol, okadaic acid (OKA), phorbol 12-myristate acetate (PMA), phosphate buffered saline tablets (PBS), phenylmethylsulfonyl fluoride (PMSF), poly-L-lysine, prostacyclin (PGI_2), Rose Bengal, sepharose 2B, sodium cacodylate, sodium citrate, sodium arachidonate, sodium dodecyl sulphate (SDS), pre-stained low range molecular weight markers were obtained from Sigma-Aldrich, Dorset, UK. Collagen, adrenaline and arachidonic acid were obtained from Bio/Data, Horsham, USA. Acetic acid, acetone, acrylamide, ammonium persulfate (APS), bis-acrylamide, chloroform, colloidal coomassie brilliant blue stain, diethyl ether, formaldehyde, glutaraldehyde, hexane, hydrochloric acid, methanol, triton X-100, N,N,N',N'-tetramethylethylendimin (TEMED), Tris(hydroxymethyl)methylamine (Tris), trichloroacetic acid (TCA), prostaglandins D_2, E_2, $F_{2\alpha}$, protease inhibitor cocktail set III, sulphuric acid was obtained from VWR International, Dorset, UK. Ecoscint A was obtained from National Diagnostics Limited (Wigan, UK). Absolute alcohol 100 purchased from Hayman Industries, Essex, UK was supplied via the university chemical stores. [^{14}C]-Arachidonic acid, chromium-51, cyclic adenosine monophosphate (cAMP) enzyme immunosorbent assay was obtained from Amersham, UK. PAC-1 FITC IgM antibody and CD42a PerCP, clone Beb1 was obtained from Becton Dickinson Biosciences UK Limited, UK. Aged Garlic Extract (Kyolic), alliin, cycloalliin, β-chlorogenin (βC), frutosylarginine (FA), S-allylcysteine (SAC), S-allylmercaptocysteine (SAMC), S-ethylcysteine (SEC), S-methylcysteine (SMC), and S-propenylcysteine (SPC) were

kindly provided by Wakunaga of America Limited (Mission Viejo, CA). NB. All solvents used were HPLC grade.

2.1.2. Aged Garlic Extract

Aged garlic extract (AGE) is produced by soaking raw garlic in 15-20 % aqueous ethanol for approximately 20 months at room temperature. The extract is then filtered and concentrated under reduced pressure at low temperature. The presence of water-soluble compounds found in AGE is relatively high compared to that of oil-soluble compounds which is low. The AGE used in this study for both *in vitro* and *in vivo* investigations contained 305 g/L extracted solids, with S-allylcysteine, the most abundant water-soluble compound, was present at 1.47 g/L. The same batch of AGE was used in both the *in vitro* and *in vivo* studies.

2.2. VOLUNTEER BLOOD COLLECTION

This study has been approved by both the Research Degrees and Ethics Committee (Liverpool John Moores University), and informed consent was obtained from all volunteers (Appendix I). Blood samples (20 mL) were collected via venepuncture from healthy volunteers who had not taken any medication known to interfere with platelet function for two weeks prior to donation, and was mixed with 3.8 % (w/v) trisodium citrate as the anticoagulant in the ratio 9:1 (v/v).

2.3. PREPARATION OF HUMAN PLATELET SUSPENSIONS

2.3.1. Preparation of human platelet-rich plasma

Platelet-rich plasma (PRP) was prepared as described previously by Rahman and Billington (2000). In brief, whole-citrated blood was centrifuged for 10 min at 150 x g. The PRP was removed and the remaining blood was centrifuged further for 20 min at 1500 x g to obtain platelet-poor plasma (PPP). The platelet count for the PRP was determined using a Neubauer Haemocytometer and the platelet count was adjusted if necessary to $2.5 \pm 0.5 \times 10^5$ cells/mL by dilution with PPP. All the samples were kept at room temperature prior to testing, and the experiments were completed within 3h of blood collection.

2.3.2 Preparation of gel-filtered platelet suspensions

Gel-filtered platelets (GFP) were prepared as described by Walkowiak *et al.* (2000). Briefly, a 11 mL packed Sepharose 2B column was washed and equilibrated in HEPES-Tyrode's buffer, pH 7.4 (138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 1 mg/mL glucose, 20 mM HEPES). Prior to platelet separation from PRP, excess buffer was removed from the top of the column. Upon the addition of the total PRP volume (1 mL) to the gel surface, HEPES-Tyrode's buffer was layered onto the column gradually in volumes required for proper column operation. Platelet suspensions, with the appearance of clouded drops (1.5 mL) were collected after an elution volume of 2.5 mL, and platelet concentration was determined using a Neubauer Haemocytometer and the platelet count was adjusted with buffer if necessary to 2.5×10^5 cells/mL.

2.3.3. Preparation of washed platelet suspensions

Washed platelet suspensions were prepared following the method developed by Radomski and Moncada (1983). In brief, PRP was supplemented with PGI_2, to the final concentration of 300 nmol/L to prevent spontaneous platelet aggregation from occurring by increasing the intraplatelet levels of cAMP. The PGI_2 treated PRP was centrifuged for 15 min at 1000 x g, the resultant platelet pellet was resuspended in PBS or HEPES-Tyrode's buffer and centrifuged again. After the washing procedure was completed the platelet pellet was resuspended in buffer and the platelet count adjusted to 2.5×10^5 cells/mL.

2.4. PLATELET AGGREGATION STUDIES

2.4.1. Measurement of platelet aggregation using a light transmittance method

Platelet aggregation was performed in a PAP-4D Platelet Aggregation Profiler (Bio/Data Corporation, Horsham, PA), which was calibrated first with PPP (100 % aggregation) followed by PRP (0 % aggregation). Aggregation was carried out in 200 µL of PRP at 37°C and initiated via the addition of 20 µL of an aggregating agent (Refer to Table 2.1.). The PAP-4D is a multi-channel aggregometer that uses a light transmission method to measure the following parameters: total percentage aggregation and the initial slope of aggregation. The parameter used throughout these studies was

total percentage aggregation. In this study ADP was used as the primary agonist unless otherwise stated.

2.4.2. Inhibition of agonist-induced platelet aggregation by AGE and its constituents

The following reagents were added to freshly prepared human PRP and incubated for 10 min at 37°C. Platelet aggregation was initiated via the addition of an aggregating agent (Table 2.1). To the controls an equal volume of the appropriate vehicle was added to the PRP and treated as above.

i. Concentrations of AGE were prepared from neat liquid AGE (100 %) by dilution in PBS and the following final percentage concentrations (v/v) in PRP were: 0.78, 1.56, 3.12, 6.25, 12.5 and 25 % (v/v), respectively.

Table 2.1. Concentrations of agonists used to initiate platelet aggregation *in vitro,* that produced maximal total percentage aggregation when measured in a platelelet aggregometer.

Agonist	Final Concentration when added to PRP (0.2 mL)
ADP	8 μmol/L
AA	250 μg/mL
Adrenaline	20 μmol/L
A23187	5 μmol/L
Collagen	190 μg/mL
Thrombin	0.1 U/L
PMA	10 μmol/L

ii. Ethanol diluted in PBS to the final concentrations: 0.1, 0.25, 0.5 and 1.0 %, was added to 0.2 mL PRP respectively. To the controls an equal volume of PBS was added to the PRP and platelet aggregation was initiated via the addition of either ADP (8 μmol/L) or collagen (190 μg/mL).

iii. Nine individual components isolated from AGE: alliin, cycloalliin, β-chlorogenin (βC), frutosylarginine (FA), S-allylcysteine (SAC), S-allylmercaptocysteine (SAMC), S-ethylcysteine (SEC), S-methylcysteine (SMC), and S-propenylcysteine (SPC) were added to PRP and incubated for 10 min at 37°C platelet aggregation was initiated with the agonist ADP. The concentrations of individual components used in the experiment are presented in Table 2.2.

The individual components listed in Table 2.2 were also mixed together and their effect upon platelet aggregation was examined. Seven mixtures were prepared; mixture 1 (M1) contained all nine components at the final concentrations in PRP that produced the greatest degree of inhibition compared to the control when tested individually (Table 2.3). This was done to try and establish if all the individual components work synergistically to inhibit platelet aggregation. The subsequent six mixtures contained all nine constituents at the following final concentrations in PRP: 0.0195, 0.78, 6.25, 100 and 250 μmol/L and 1.0 mmol/L. These concentrations were used as it is not known what the exact concentration of each constituent is within AGE.

Table 2.2 Final concentration range of each individual component from AGE added to PRP

Components	Concentration range of each component when added to PRP (μmol/L)
Alliin	
Cycloalliin	
βC	**0.78**
FA	**3.125**
SAC	**6.25**
SAMC	**25**
SEC	**100**
SMC	
SPC	

Table 2.3. Concentrations of individual AGE components contained within mixture 1 (M1). Concentration used in M1 was capable of inhibiting platelet aggregation when each component was examined individually.

Individual component from AGE	**Concentration that produced most inhibition when tested on its own (μmol/L)**
Alliin	100
βC	6.25
Cycloalliin	3.125
FA	0.78
SAC	0.78
SAMC	3.125
SEC	0.78
SMC	6.25
SPC	6.25

iv. The amino acids L-arginine, L-cysteine and L-methionine are found within AGE. These amino acids were tested both individually and as a mixture for their possible inhibitory effects upon ADP-induced platelet aggregation *in vitro*. The following final concentrations were added to PRP: 0.195, 0.78, 6.25, 100 μmol/L and 1.0 and 9.0 mmol/L.

v. The amino acids: L-arginine, L-cysteine and L-methionine and the nine isolated components from AGE: alliin, cycloalliin, β-chlorogenin (βC), frutosylarginine (FA), s-allylcysteine (SAC), s-allylmercaptocysteine (SAMC), s-ethylcysteine (SEC), s-methylcysteine (SMC), and s-propenylcysteine (SPC) were mixed together and the effect of this mixture upon platelet aggregation was examined. The following final concentrations of each individual component were mixed together and added to PRP: 0.195, 0.78, 6.25, 100, 250 μmol/L and 1.0 mmol/L, respectively.

2.4.3. Classical inhibitors of platelet aggregation

Table 2.4 shows the classical inhibitors of platelet aggregation and the concentrations used in platelet aggregation studies. The effects of the various inhibitors were compared to that of AGE (25 % v/v). The inhibitory actions of each classical inhibitor is summarised in Table 2.5.

Aspirin was diluted in NaCl, 0.9 % (w/v) and was used to make up the concentrations listed in Table 2.4. bisindolymalemide (BIS), genistein (GEN), 3-isobutyl-1-methylxanthine (IBMX), Indomethacin, okadaic acid (OKA) and prostaglandin E_1 (PGE_1) were dissolved in DMSO, and diluted with PBS to the required concentration for the aggregation study (Table 2.4), the concentration of DMSO was kept below 0.05 % (v/v). Both AlF_3 and NaF were diluted with PBS.

Table 2.4 Classical inhibitors of platelet aggregation. The following concentrations of inhibitors were added to PRP and incubated for 10 min at 37°C, prior to activation with the agonist ADP.

Classical inhibitors of platelet aggregation	Concentrations used in platelet aggregation studies (μmol/L)
Aspirin (acetylsalicyclic acid)	0.195, 0.78, 6.25, 80, 100, 250, 500 μmol/L, and 1.0 mmol/L
AlF_3	100
AG527	35
BIS	100
GEN	100
IBMX	150
Indomethacin	3.9, 16, 62.5, 250 μmol/L and 1.0 mmol/L
NaF	100
OKA	4
PGE_1	7.5, 15, 31.25, 62.5, 125 and 250 μmol/L

Table 2.5. Classical inhibitors of platelet aggregation and their mechanism of inhibition.

Classical Inhibitor	Mechanism of Inhibition
AlF_3	G-proteins
AG527 - Tyrphostin	Protein tyrosine kinases
BIS	Protein kinase C
GEN	Protein tyrosine kinases
IBMX	cAMP and cGMP phosphodiesterases
Indomethacin	Cyclooxygenase inhibitor
OKA	Protein phosphatases
NaF	Ca^{2+} mobilisation
PGE_1	Increases intracellular cAMP levels

2.4.4. AGE and its disaggregatory effect on agonist-induced platelet aggregation

To examine the disaggregatory effects of AGE, it was added at various concentrations (% v/v) to PRP that had been preaggregated with the agonists: ADP, collagen and thrombin.

The experiment was conducted only if total percentage aggregation (%) was observed to be ≥ 60 % upon the addition of an agonist to PRP in the platelet aggregometer. AGE at the following final concentrations: 0.78, 1.56, 3.125, 6.25, 12.5 and 25 % (v/v), respectively, was added to platelets that had undergone aggregation following agonist stimulation with ADP (8 μmol/L), collagen (190 μg/mL) or thrombin (0.1 U). The disaggregatory effect (dispersal of aggregated platelets) was measured as the reduction in total percentage aggregation compared to a PBS control, using the formula reported by Naimushin and Mazurov (2003).

$$[(C-A)/C)] \times 100 = DA \text{ (disaggregation effect)}$$

C – PBS control

A – AGE treated platelets

2.5. THE EFFECT OF AGE ON PLATELET INTRACELLULAR SIGNALLING PATHWAYS INVOLVED IN PLATLET ACTIVATION AND AGGREGATION IN VITRO

2.5.1. The effect of AGE on intracellular calcium signalling in platelets

Intracellular Ca^{2+} measurement was carried out using the method of Park *et al.,* (2004). In brief, Fura-2/AM (a fluorescent marker that binds to the calcium ion) was added to PRP at a final concentration of 1 μmol/L and incubated for 45 min at 37°C. Fura-2/AM loaded platelets were washed by centrifugation with HEPES-Tyrode's buffer and resuspended in HEPES-Tyrode's buffer (see section 2.2.2) at a concentration of 2.5 x 10^6 cells/mL. Washed platelets containing Fura-2/AM were incubated with either AGE or a diethyl extract of AGE at a final concentration of 5 % (v/v), respectively for 10 min at 37°C. These experiments were performed in the presence of 1 mM $CaCl_2$, 1 mM $CaCl_2$ plus 1 mM EGTA or in the presence of EGTA alone prior to the addition of the agonists A23187, a calcium ionphore (5 μmol/L) or ADP (8 μmol/L). Fluorescence was

measured using the ratio scan function on a VARIAN Cary Eclipse Fluorescence Spectrophotometer (Australia) and its software, version 1.1(132). The ratio scan was performed over a 3 min time period using two excitation wavelengths alternating between 340 and 380 nm every 0.5 seconds, with the emission wavelength set to 510 nm, respectively. The calcium concentration was calculated for the first 2 min of the scan using the following equation developed by Grynekiwicz *et al.* (1985):

$$[Ca^{2+}] = kd \times \frac{Rt-Rmin}{Rmax-Rt} \times \frac{Sf(\lambda 2)}{Sb(\lambda 2)}$$

kd – the effective dissociation constant (135.00)

Rmin – the limiting value that R can have at zero $[Ca^{2+}]$

Rmax - the ratio that has saturating $[Ca^{2+}]$

Rt – fluorescence ratio - F_1/F_2

S – the product of the excitation intensity (f – free dye measured at wavelength λ2; b – calcium bound dye measured at wavelength λ2)

2.5.2. The effect of AGE on the arachidonic acid metabolic pathway in platelets

Arachidonic acid uptake was monitored using the method outlined by Jin *et al.*, (2002). In brief, PRP containing approximately 2×10^8 cells/mL was incubated at 37°C for 20 min with 1 μCi ^{14}C labelled arachidonic acid in PBS. The labelled platelets were washed and resuspended in modified HEPES-Tyrode's buffer containing: 137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 5 mM glucose, 10 mM HEPES, pH 7.4 and 0.2 % (w/v) BSA. Aliquots (500 μL) of the radiolabelled platelet suspension were activated with the agonists ADP or collagen that had been pre-incubated for 10 min at 37°C in the presence or absence of 3.12 % (v/v) AGE. 100 μL aliquots were taken at the following time points: 0, 0.5, 1.0 and 3 min after agonist stimulation. Platelet aggregation was terminated via the addition of one-quarter volume of stopping solution containing 2 % formaldehyde and 40 mM EDTA resulting in the rupture of the platelet membranes. Samples were collected and centrifuged at 5000 x g for 1 min and the supernatant was collected into scintillant (Ecoscint A) (National Diagnostics, UK) and the radioactivity (ß emissions) measured using a Beckman LS 6500 multi-purpose liquid scintillation counter (USA). To the controls PRP labelled with cold AA was compared to an equal amount of ^{14}C-AA contained within scintillant.

2.5.3. AGE and its effect upon fibrinogen binding in ADP-induced platelet aggregation

The effect of AGE upon fibrinogen binding was monitored using a simple adhesion assay (Figure 2.1). The final step in platelet aggregation *in vivo* common to all forms of agonist stimulation is the binding of the fibrinogen molecule to its receptor GPIIb/IIIa. This binding allows adjacent activated platelets to cross-link with each other to form a platelet aggregate. Thus, it is important to observe the effects of AGE on this process *in vitro.* This simple assay allows this by observing the effects of AGE-treated platelets and their ability to bind to immobilised fibrinogen when stimulated with an agonist such as ADP.

A 96-well microtitre plate was coated with fibrinogen (3 g/L) overnight at 4°C. Excess fibrinogen was removed from the wells and non-specific binding sites were blocked with 1 % BSA in PBS buffer. PRP was pre-treated with AGE (0.78-25 % v/v) for 10 min at 37°C. The AGE treated platelets were added to the fibrinogen coated wells and aggregation was initiated via the addition of ADP and the reaction was allowed to run for 5 min. The reaction was stopped by placing the microtitre plate onto ice. The non-adherent platelets was removed from each well via aspiration and was followed by 3 x 200 µL PBS washes. 200 µL of 0.2 % (w/v) Rose Bengal was added to the wells for 30 min at room temperature. The Rose Bengal was removed and the wells washed again 3 x 200 µL with PBS. The adherent platelets were lysed with lysis buffer (150 mM NaCl, 50 mM NaF, 13 mM $Na_4P_2O_7.10H_2O$, 1.1 mM Na_3SO_4, 5 mM EDTA, 10 mM Tris, pH 7.4, 1 % Triton X-100). To the primary controls AGE was replaced with an equivalent volume of PBS and treated as above. To the secondary controls the platelets were not stimulated with ADP and to the tertiary controls, no Rose Bengal was added to the platelets. The released Rose Bengal from the platelet cytosol was measured using a Titertek Multiskan MCC/340 MK II plate reader with a 540 nm filter.

In order to validate the Rose Bengal data, the experiment was repeated with chromium-51 (5 µCi/mL platelets). Excess chromium was removed via gel filtration (Section 2.2.2). The released chromium was measured via liquid scintillation (Ecoscint A) using a Beckman LS 6500 multi-purpose scintillation counter (USA).

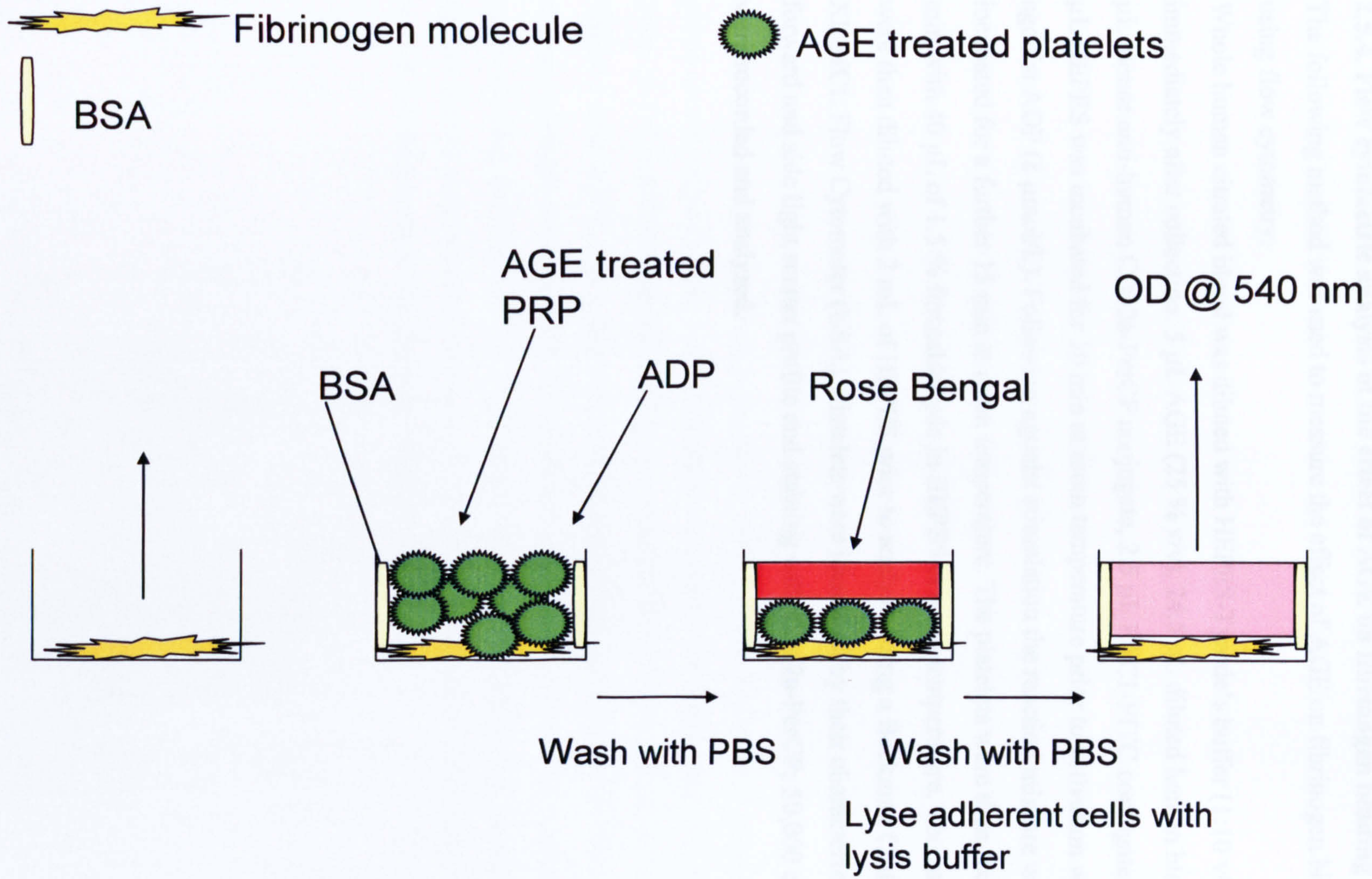


Figure 2.1. Principles of fibrinogen adhesion assay. Microtitre wells are coated with fibrinogen overnight at 4°C. Excess fibrinogen is removed and 1 % BSA is added to wells to prevent non-specific binding from occurring. PRP is incubated with AGE, and the platelets are added to the wells and aggregation initiated with ADP. The non-adherent platelets are removed, the wells washed with PBS and Rose Bengal is added to the adherent platelets. After a set incubation time the Rose Bengal is removed, the wells washed and the adherent platelets are lysed. The lysed platelets release Rose Bengal from their cytosol and this can be monitored by measuring the optical density using a 540 nm filter.

2.5.4. Flow cytometric analysis of the effect of AGE on fibrinogen binding

The following method was used to measure the effect of AGE on fibrinogen binding using flow cytometry:

Whole human citrated blood was diluted with HEPES-Tyrode's buffer (1:10 v/v) immediately after collection. 5 µL AGE (25 % v/v), 24.5 µL diluted human blood, 1.5 µL mouse anti-human CD42a-PerCP conjugate, 2.5 µL PAC1-FITC conjugate and 1.5 µL HEPES was incubated for 10 min at room temperature prior to activation with the agonist ADP (8 µmol/L). Following agonist stimulation the reaction mixture was incubated for a further 15 min at room temperature. The platelets were then fixed for 30 min with 40 µL of 1.5 % formaldehyde in HEPES at room temperature. The samples were then diluted with 2 mL of HEPES prior to analysis using a Beckman Coulter Epics XL.MCL Flow Cytometer (USA). Platelets were identified by their characteristic forward and side light scatter profile and staining with CD42a-PerCP; 50,000 events were recorded and analysed.

2.5.5. cAMP enzyme immunosorbent assay

Intraplatelet cAMP levels were measured using a competitive enzyme immunosorbent assay (Figure 2.2).

Briefly, citrated PRP was incubated with AGE (25 % v/v, final concentration), PGE_1 (10 μmol/L, final concentration) or an equivalent volume of PBS (negative control) for 10 min at 37°C, respectively. ADP at a final concentration of 8 μmol/L was added to the platelets to initiate platelet aggregation. Aliquots were taken prior to ADP stimulation (basal) and at the following time points immediately after agonist activation: 0, 30 and 300 seconds and placed on ice. The samples were centrifuged at 1500 x g for 5 min at 4°C, and the resulting platelet pellet was resuspended in lysis buffer (0.5 mL) (provided with the kit) and left for 20 min. Intracellular cAMP levels were measured using a Biotrak enzyme immunoassay kit, following the manufacturer's instructions; 100 μL of sample or standards (range 0-3200 fmol/well) were incubated in 96 well microtitre plates coated with a Donkey anti-rabbit antibody specific for Rabbit anti-cAMP for 2 hours at 4°C. cAMP-peroxidase conjugate was added to the wells and incubated for a further 1 hour at 4°C. The wells were washed thoroughly and incubated with the enzyme substrate solution, TMB for approximately 20 min at room temperature with gently shaking. Sulphuric acid (1.0 M) was added to the wells as a stop solution and the optical density was measured immediately at 450 nm using a microtitre plate reader. A typical standard curve is displayed in Appendix II.

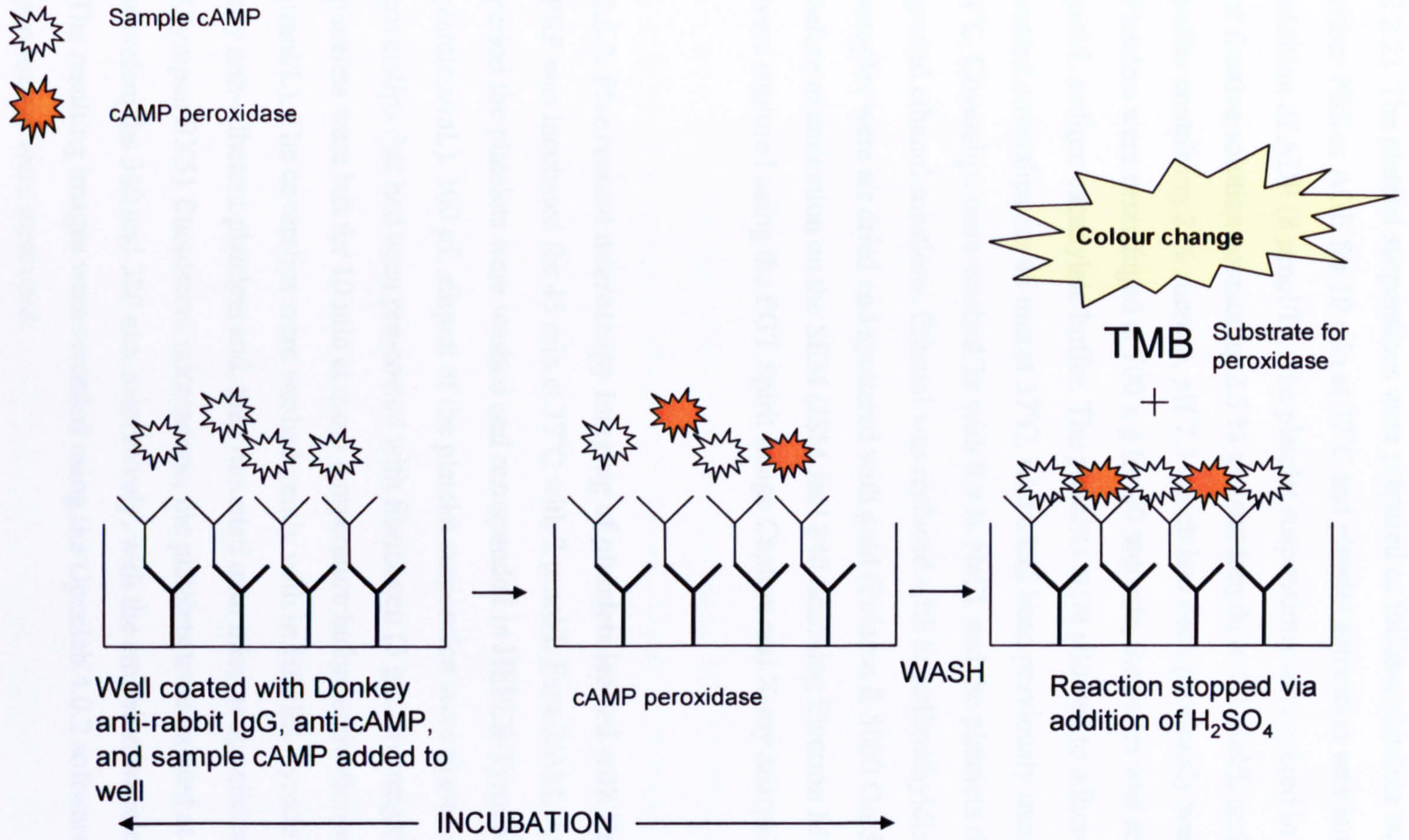


Figure 2.2. The principles of cAMP Competitive Enzyme Immunoassay. The Rabbit anti-cAMP antibody binds to the wells coated with Donkey anti-rabbit antibody. There is competition between the sample cAMP and the peroxidase conjugated cAMP for binding to the Rabbit anti-cAMP antibody. The TMB substrate is added for colour development to occur. The amount of colour that is developed is inversely proportional to the amount of cAMP within the sample.

2.6. IMAGING STUDIES OF PLATLET AGGREGATION

2.6.1 Scanning Electron Microscopy imaging of platelet aggregation

PRP was washed and resuspended in HEPES-Tyrode's buffer, pH 7.4 (see section 2.2.2). The platelet suspensions were prepared as follows platelets were incubated with either PBS or AGE for 10 min at 37°C and platelet activation was induced via the addition of ADP (8 μmol/L). The platelet suspensions were mixed in an equal volume of fixative solution containing 2.5 % glutaraldehyde in 0.1 mol/L sodium cacodylate buffer containing 2 % sucrose, pH 7.3 which had been previously warmed to 37°C. Platelets were centrifuged at 700 x g for 20 seconds, the pellet was resuspended in 0.1 mol/L sodium cacadylate buffer. The platelets were allowed to adhere to poly-$_L$-lysine coated coverslips for 45 min at 37°C, which had been previously incubated overnight at 4°C. Coverslips were washed 3x with 0.9 % NaCl, and the platelets dehydrated in graded ethanol solutions. Ethanol was replaced with hexadimethyldisilazane and the samples were air dried and sputtered with gold (Polaron E 5000 Gold Sputter Coater) before examination on the SEM (JSM Jeol 840 Scanning Electron Microscope). Images were captured using the PGT Spirit Image Capture and X-ray analysis software.

2.6.2. Fluorescent microscopy imaging of platelets loaded with Fura-2/AM

PRP was incubated for 45 min at 37°C with 2 μmol/L Fura-2/AM. After the incubation period the platelets were washed and resuspended in HEPES-Tyrode's buffer (2 x 10^6 platelets/mL). 100 μL aliquot of the platelet suspension were placed onto glass coverslips that had been pre-coated with fibrinogen (3 g/L) overnight at 4°C. The platelets were left for 10 min at room temperature before activation with ADP (8 μmol/L). The coverslips were washed gently with in HEPES-Tyrode's buffer to remove any non-adherent platelets and, then mounted onto microscope slides. Using an Olympus BX51 fluorescent microscope, the platelets were excited at the following wavelengths 340 and 380 nm, respectively, with the emission wavelength set at 510 nm. The resulting images were recorded using the Openlab 4.0.2 software. The following parameters were measured:

1. Platelets activated with ADP
2. Platelets pre-treated with AGE and activated with ADP.

2.7. PROTEOLYTIC ACTIVITY OF AGED GARLIC EXTRACT

2.7.1. Protease assay to measure the proteolytic activity of AGE

The proteolytic activity of AGE was assessed using a modified version of the method outlined by Brock *et al.* (1982). In brief, samples were prepared as follows: 500 µL of 300 mM Tris-HCL buffer, 50 µL of AGE (3.12, 6.25, 12.5, 25 and 100 % v/v, final concentrations) diluted in PBS and 500 µL of azocasein solution (10 mg/mL in distilled water) as the enzyme substrate was mixed thoroughly, and incubated for 0-18 hours at 37°C. The reaction was terminated at specific time points via the addition of 70 % (w/v) TCA (final concentration 6 %), vortexed and placed on ice for 30 min. This was followed by centrifugation for 5 min at 3000 x g. The resulting supernatant was removed and an aliquot (500 µL) was placed into a cuvette containing 1 M NaOH. The optical density was measured at 450 nm in a Uvicon 930 dual beam spectrophotometer (Kantron Instruments, USA), against a blank containing Tris-HCL buffer, azocasein solution and water as a replacement for the AGE.

The optical density readings generated in this study were compared to a standard curve (Appendix III) of a range of chymotrypsin standards (1,2,4,6,8,10,20,40,60,80 and 100 µg/mL) a known protease specific for the protein casein. Chymotrypsin standards were prepared with distilled water and treated as the above. The standard curve was used to determine if AGE displayed proteolytic activity similar to that of chymotrypsin.

2.7.2. Protein concentration determination in AGE using the Bradford assay

The concentration of protein present in AGE was determined using the method of Bradford (1976). In brief, 100 µL of AGE (3.12-100 %, v/v diluted in PBS) was added to 0.25 mL NaOH and 5.0 mL Bradford reagent, vortexed and left to incubate at room temperature for 5 min. The optical density of each AGE sample was determined at 595 nm. The protein concentration for each AGE sample was determined by comparing it to a BSA standard curve (Appendix IV). The BSA samples used were: 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.25. 0.5, 1.0, 1.5 and 2.0 mg/mL, diluted in PBS.

2.8. THE EX VIVO EFFECT OF AGE ON PLATELET AGGREGATION

Whole human citrated blood was incubated with AGE at various concentrations (12.5-50 % v/v) for approximately 20 min at 37°C. The platelets were removed from the whole blood via centrifugation at 150 x g for 10 min at room temperature. The platelet count was adjusted if necessary with PBS/PPP to $2.5 \pm 0.5 \times 10^5$ platelets/mL. Aggregation was initiated with ADP (8 μmol/L) and the aggregation curves were monitored using a platelet aggregometer. To the control sample an equivalent volume of PBS was added to the whole blood and treated in the same manner as above.

2.9. CLINICAL TRIAL – DIETARY SUPPLEMENTATION WITH AGE

Volunteers for the clinical trial were recruited from the university population (n=20, age range 22-62 yrs) who appeared to be apparently healthy and had not taken any medication known to interfere with platelet function 2 weeks prior to the start of the trial. Informed consent was obtained from all volunteers before the start of the trial (Appendix V). All volunteers answered a short questionnaire (Appendix VI) regarding their age, gender, weekly alcohol consumption, daily cigarette intake, health conditions, prescribed medication and other dietary supplement intake. Volunteers consumed 5 mL of AGE daily for 2 weeks at set times throughout the day, usually in the morning. The dose of AGE used in this trial is that recommended by the manufacturer's of AGE, and has been shown previously to be beneficial in the reduction of platelet aggregation following AGE ingestion (Rahman and Billington, 2000). The volunteers were asked to follow their usual diet and lifestyle throughout the study. Venous blood samples (20 mL) were taken before the ingestion of AGE (baseline), after 2 weeks of AGE ingestion and following a 2 week washout period were no AGE was consumed at all.

The following parameters were measured during the trial:

- Platelet aggregation initiated with the agonists: ADP, AA, adrenaline and collagen (see section 2.3.1.).
- GPIIb/IIIa receptor binding using flow cytometry (see section 2.5.4.).
- cAMP levels (see section 2.4.4.)
- Total cholesterol analysis

2.10. STATISTICAL ANALYSIS

Experimental results are expressed as the means ± SEM and are accompanied by the number of observations. Data was assessed using the student's paired t-test (Microsoft Excel ToolPak, Statistical Software Add-on). A *P*-value of less than 0.05 was considered statistically significant.

CHAPTER 3
THE EFFECT OF AGED GARLIC EXTRACT AND ITS CONSTITUENTS ON AGONIST-INDUCED PLATELET AGGREGATION *IN VITRO*

3. THE EFFECT OF AGED GARLIC EXTRACT AND ITS CONSTITUENTS ON AGONIST-INDUCED PLATELET AGGREGATION *IN VITRO*

3.1 INTRODUCTION

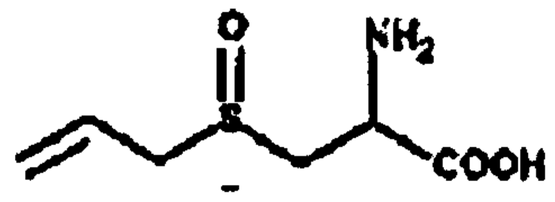
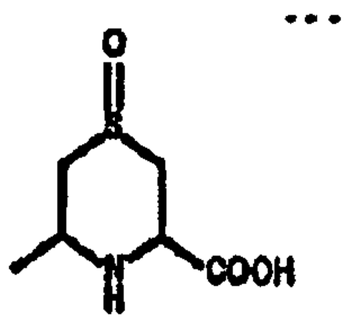
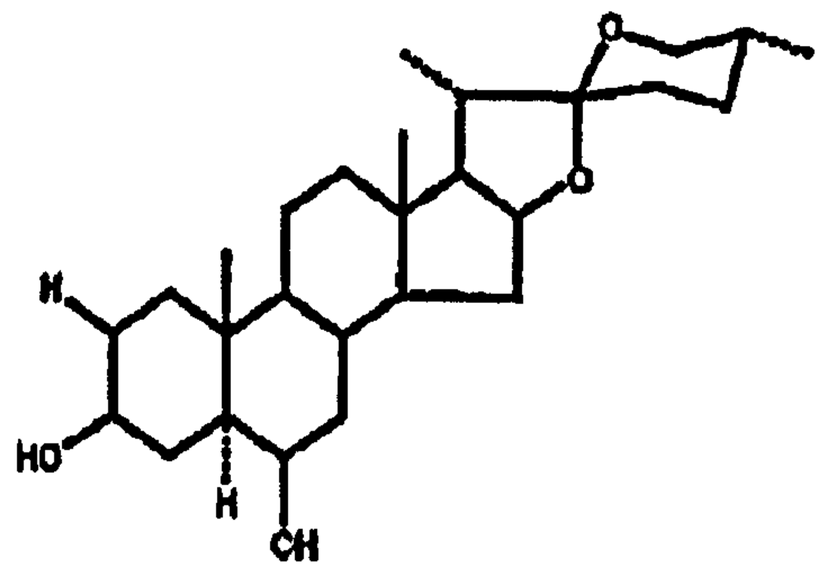
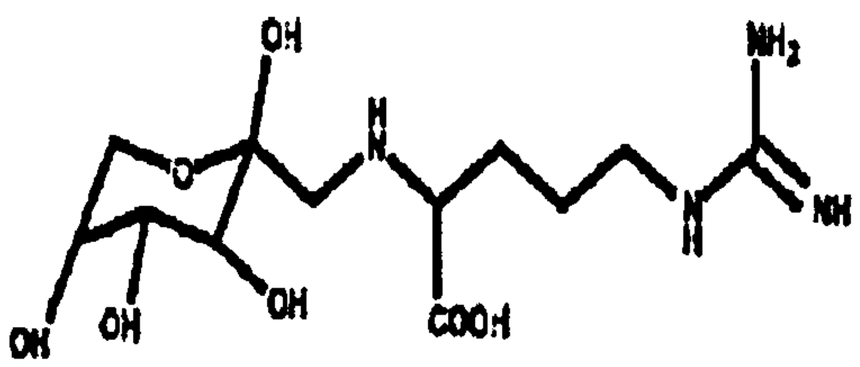
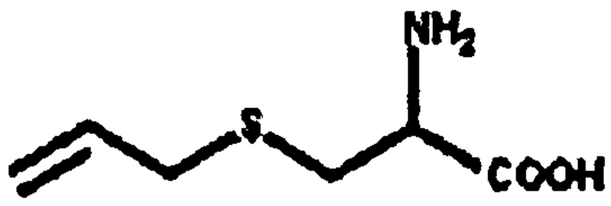
Platelet aggregation is a complex process that requires the co-operation of many biochemical pathways. Before platelets can aggregate they need to be activated by agonists such as ADP (adenosine diphosphate), AA (arachidonic acid), collagen and adrenaline etc. The major physiological agonists are collagen and thrombin, and these agents are referred to as strong agonists because they are the primary agonists *in vivo.* The aggregating agents ADP and adrenaline are known as weak agonists, as in normal platelet aggregation processes they do not normally induce platelet aggregation; instead they aid the stronger agonists by amplifying their initial responses (Wilde *et al.* 2000). ADP and adrenaline are stored within the dense granules of platelets, and upon activation the contents within these granules are released extracellularly and act in both an autocrine and paracrine manner, via specific receptors on the platelet surface or adjacent platelets, and this then leads to further activation and aggregation (Jackson 2001; Puri 1998; Solet *et al.* 2001). ADP also assists platelet adhesion and the formation of a platelet aggregate. It is well established that AGE, when added to platelets both *in vitro* and *in vivo* reduces the ability of the platelets to aggregate when challenged with agonists, particularly ADP and collagen (Allison *et al.* 2006; Steiner and Li 1998; Rahman and Billington 2000; Steiner and Li 2001).

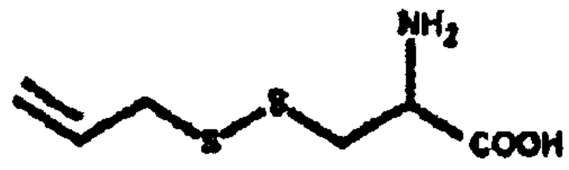
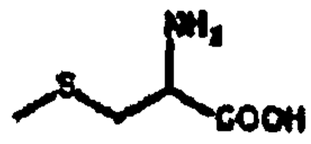
In this study, the aim was to examine the effects of AGE and some of its constituents: (Table 3.1) on their ability to inhibit platelet aggregation initiated by the agonist ADP. The major difference between fresh garlic and AGE is caused by the aging and extraction processes. In AGE there is a high abundance of water-soluble organosulphur compounds which are result of the hydrolysis of γ-glutamyl-S-allylcysteine compared to the content of raw garlic. There are also compounds unique only to AGE as a direct result of the aging and extraction process. The primary focus of this investigation was to validate previous platelet aggregation work with AGE and, to identify a possible mechanism for the inhibitory actions of AGE. This was achieved by comparing the effects of AGE to known classical inhibitors of platelet aggregation e.g. indomethacin, PGE_1 etc. All these experiments were carried out *in vitro* using the platelet aggregometry method.

3.1.2. Overview of study

Measurement of platelet aggregation in the laboratory can be achieved by a number of different methods. In this study the effects of AGE on agonist-induced platelet aggregation was monitored by utilising a light transmittance method, whereby, PRP was incubated with various concentrations of AGE or its constituents for 10 min at 37°C. Aggregation was initiated via the addition of agonists such as ADP, collagen or the calcium ionophore A23187. The principle behind this method is that as light passes through the cuvette any platelet aggregates that are formed will increase the amount of light that can pass through the cuvette. The more light that passes through the cuvette, the higher the percentage aggregation value indicating that platelet aggregation has occurred and platelet aggregates have been formed. If more light passes through the cuvette via incubation with AGE or its constituents this is an indication that inhibition of platelet aggregation has occurred, for detailed methods used in this study see chapter 2, section 2.4, page 31.

Table 3.1. Isolated compounds found in AGE; physical properties and chemical structures.

Constituent	Physical property	Structure
Alliin	s-allylcysteine sulphoxide, biosynthesied from its parent compound, γ-glutamyl-s-allylcysteine	
Cycloalliin	A sulphur containing amino acid which is not transformed during the aging process	
βC	A saponin – a compound such as this distinguishes garlic from other allium vegetables	
FA	An antioxidant not present in either heat treated or raw garlic, a unique product due to the aging process	
SAC	Unique to AGE, used for standardisation	

SAMC	Water soluble cysteine sulphoxides produced in excess during the aging process via the hydrolysis of the parent compound γ-glutamyl-s-allylcysteine	
SEC		
SMC		
SPC		

3.2. RESULTS

3.2.1 The effect of AGE and its constituents on agonist-induced platelet aggregation *in vitro*

3.2.1.I. Effect of AGE on agonist induced platelet aggregation

PRP (platelet-rich plasma) (2.5 x 10^5 platelets/mL) was incubated with various concentrations of AGE 0.78-25 % (v/v) for 10 min at 37°C. Aggregation was initiated by the addition of the following agonists (for concentrations used see table 2.1, Chapter 2, page 33): ADP (82 % ± 9.1), AA (72 % ± 5.0), adrenaline (77 % ± 12.0), A23187 (83 % ± 7.3), collagen (77 % ± 5.8), thrombin (81 % ± 5.0) and PMA (81 % ± 6.0), respectively (Figures 3.1-3.7, respectively). AGE at all concentrations had a greater inhibitory effect upon platelet aggregation when aggregation was initiated with the agonists ADP (Figure 3.1), adrenaline (Figure 3.3) and thrombin (Figure 3.6). A reduction in platelet aggregation was evident for the majority of agonists when platelets were preincubated with AGE at a concentration of 1.56 % (v/v), however this was not the case for collagen (Figure 3.5) as a higher concentration of AGE (6.25 %, v/v) was required before inhibition of platelet aggregation was statistically significant ($P<0.05$) when compared to the control .AGE dose-dependently inhibited platelet aggregation induced by the following agonists: ADP, adrenaline, collagen and thrombin. PMA (phorbol 12-myristate acetate) when added to platelets induces the stimulation of the enzyme PKC (protein kinase C), an important cell signalling molecule common to many signalling pathways; BIS (bisindolymaleimide) inhibits the action of this enzyme. BIS was used in this experiment to ensure that the PMA was able to induce platelet aggregation via PKC. From these experiments the IC_{50} values for the effect of AGE on each agonist was determined (Table 3.2). It was noted from these experiments that the agonists AA, A23187 and collagen had higher IC_{50} values (>6.25 % v/v) when compared with the remaining agonists (IC_{50} = 3.12 % AGE).

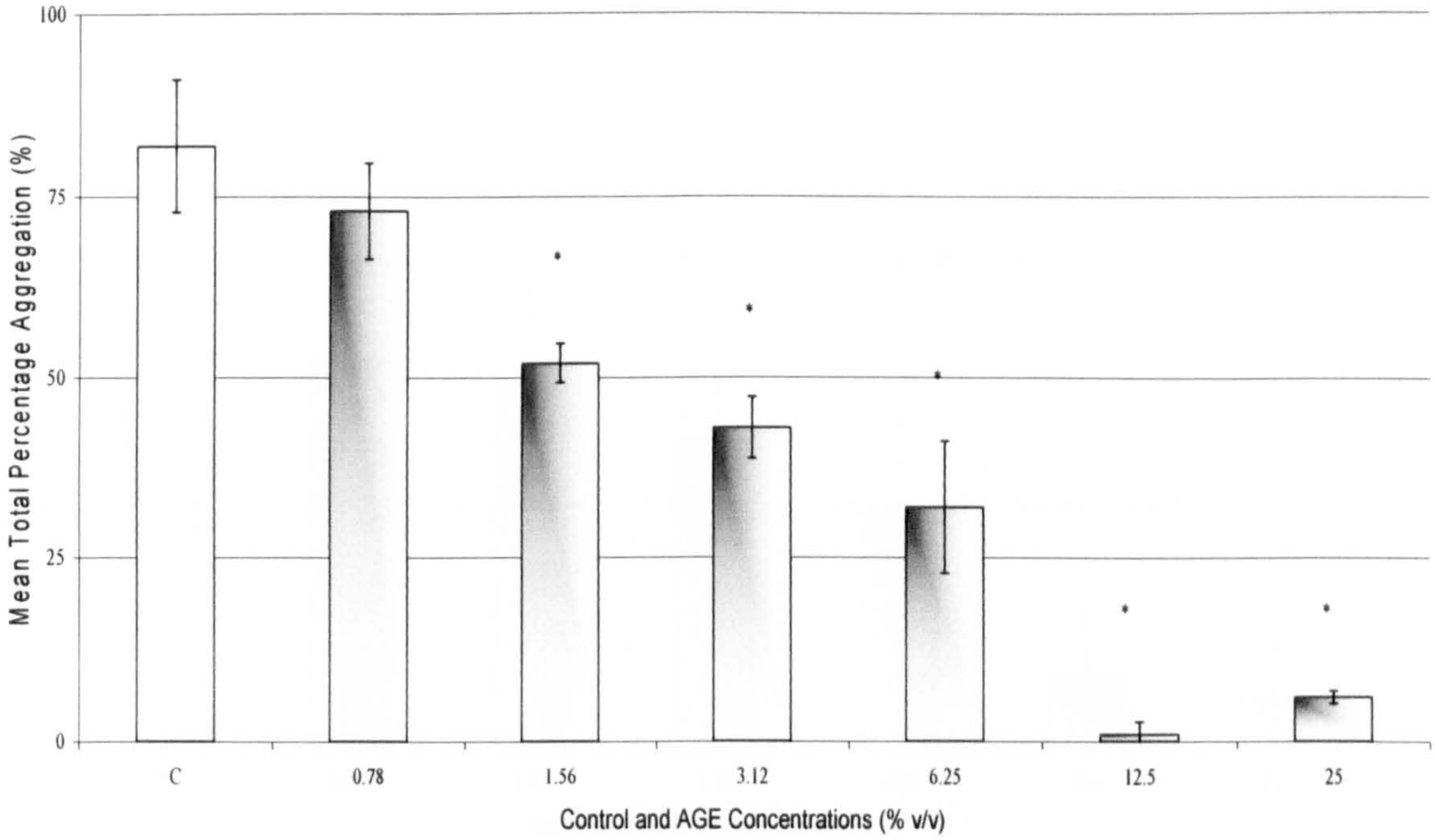


Figure 3.1. AGE and its effect upon ADP-induced platelet aggregation *in vitro*. PRP was incubated with various AGE concentrations for 10 min at 37°C. Aggregation was initiated with ADP (8 μmol/L). Aggregation curves were analysed for total percentage aggregation. Values are means ± SEM, n=6. *Statistically significant, $P<0.05$, **$P<0.01$, compared to the PBS control.

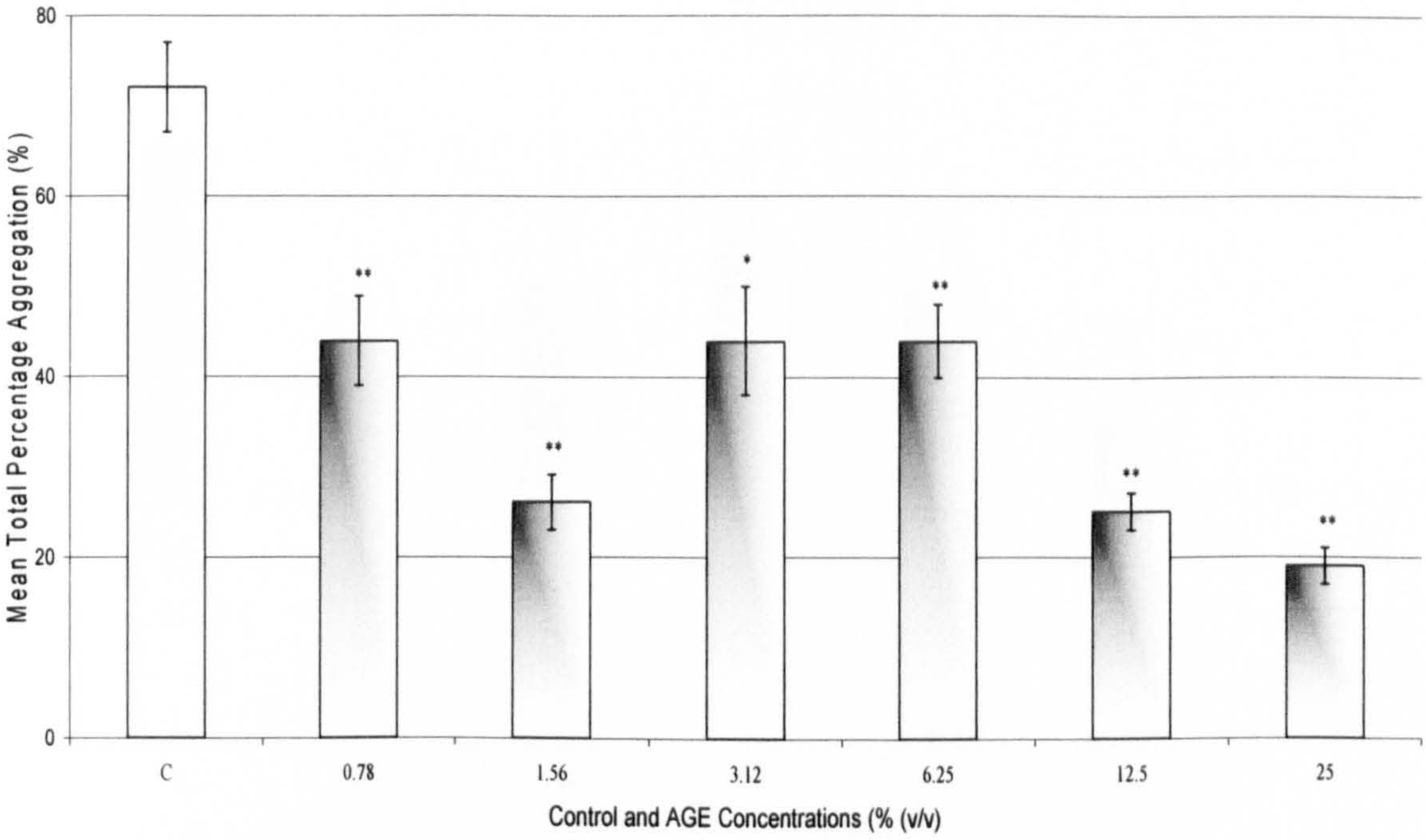


Figure 3.2. AGE and its effect upon AA-induced platelet aggregation *in vitro*. PRP was incubated with various AGE concentrations for 10 min at 37°C. Aggregation was initiated with AA (250 μg/mL). Aggregation curves were analysed for total percentage aggregation. Values are means ± SEM, n=6. *Statistically significant, $P<0.05$, **$P<0.01$, compared to the PBS control.

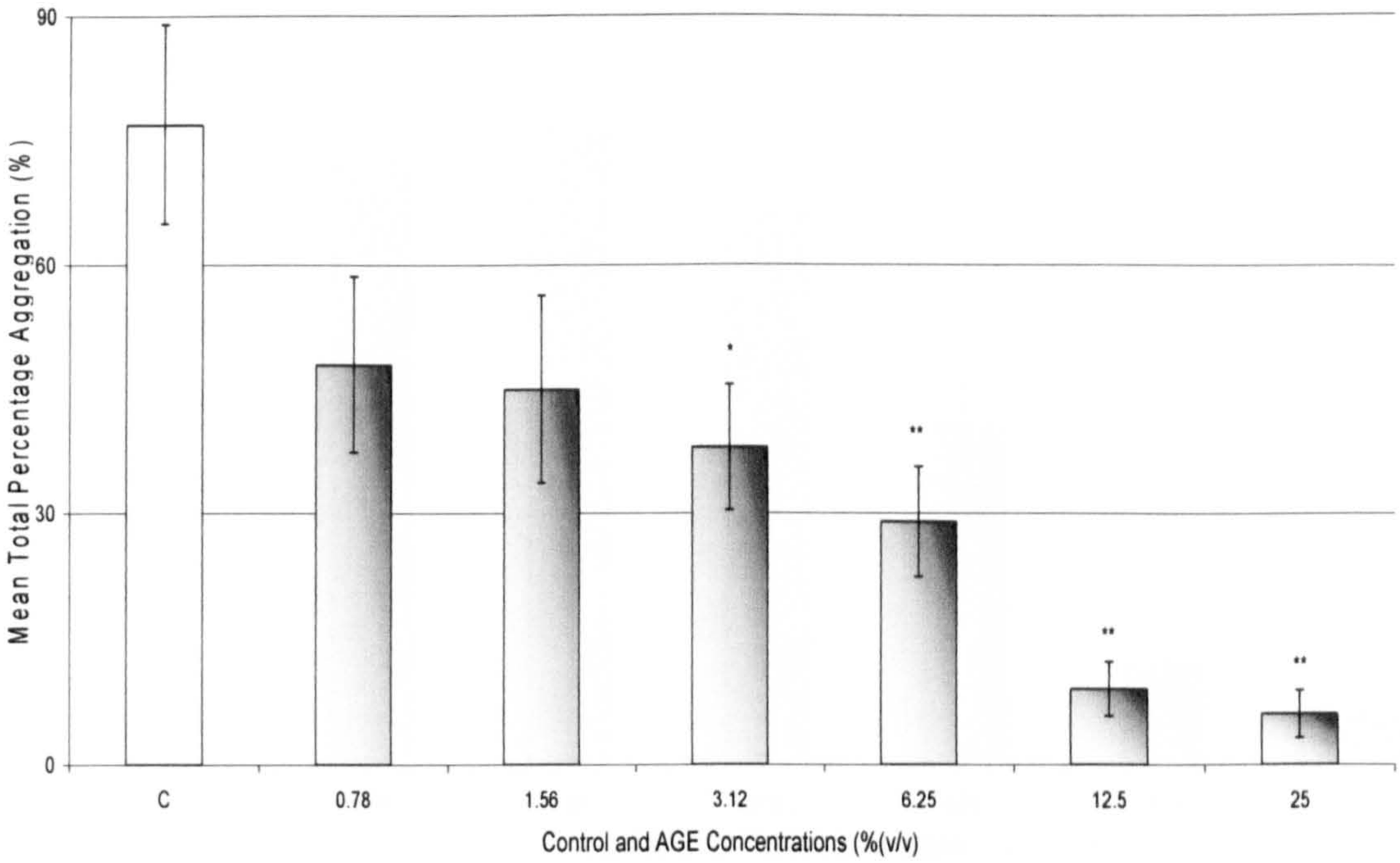


Figure 3.3. AGE and its effect upon Adrenaline-induced platelet aggregation *in vitro*. PRP was incubated with various AGE concentrations for 10 min at 37^0C. Aggregation was initiated with Adrenaline (20 μmol/L). Aggregation curves were analysed for total percentage aggregation. Values are means ± SEM, n=6. *Statistically significant, $P<0.05$, **$P<0.01$ compared to the PBS control.

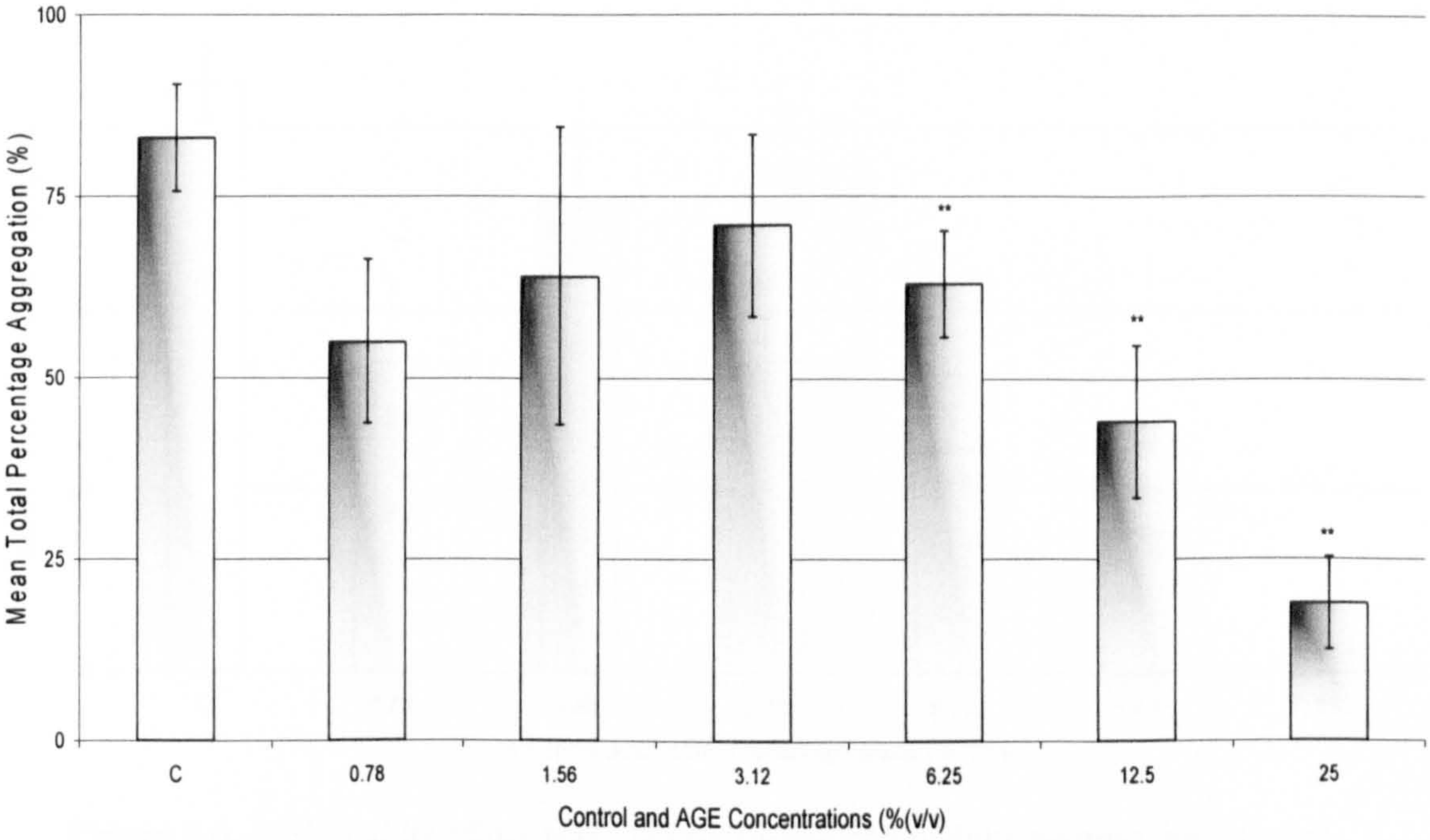


Figure 3.4. AGE and its effect upon A23187-induced platelet aggregation *in vitro*. PRP was incubated with various AGE concentrations for 10 min at 37°C. Aggregation was initiated with A23187 (5 μmol/L). Aggregation curves were analysed for total percentage aggregation. Values are means ± SEM, n=6. **Statistically significant, $P<0.01$ compared to the PBS control.

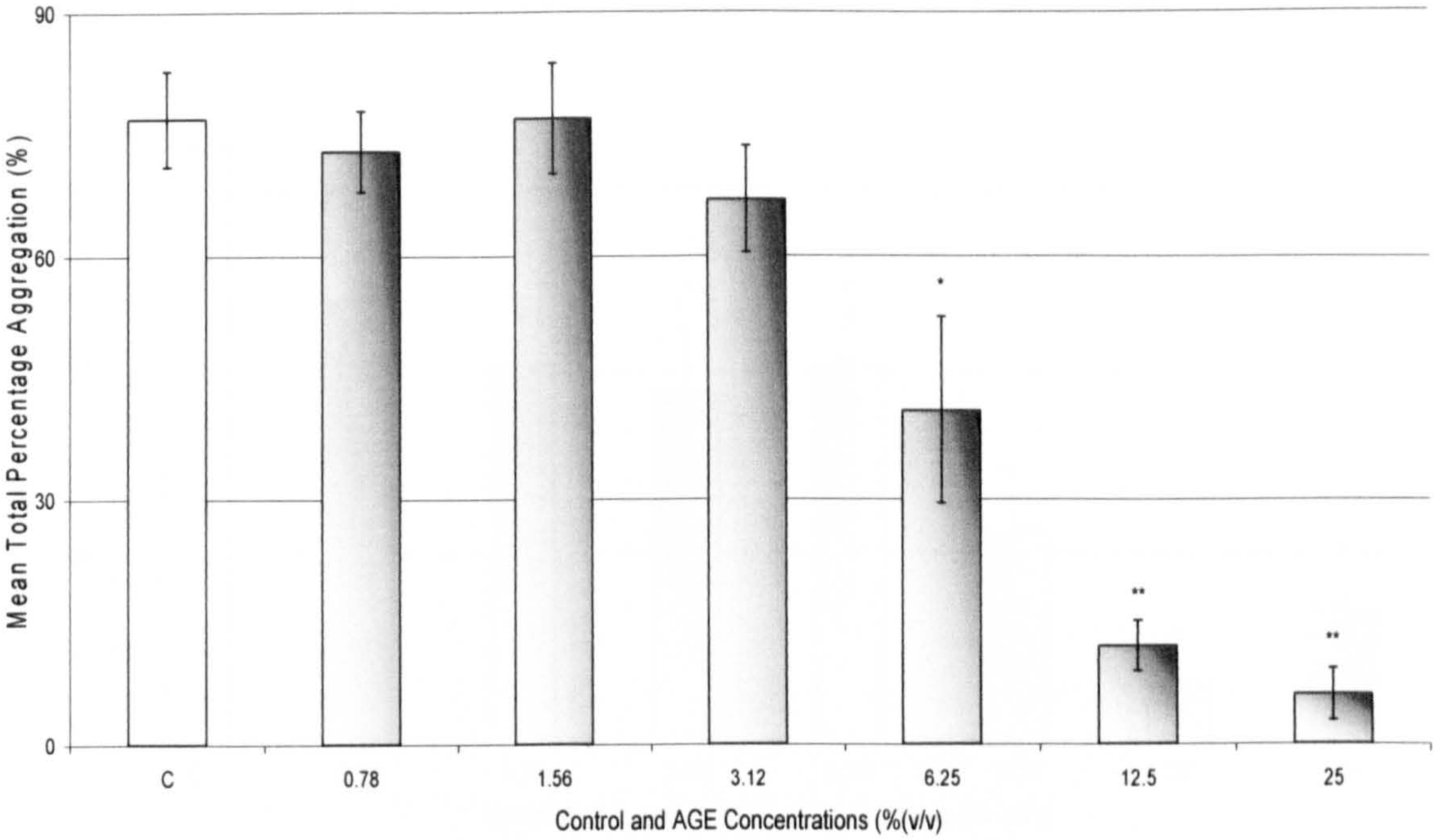


Figure 3.5. AGE and its effect upon collagen-induced platelet aggregation *in vitro.* PRP was incubated with various AGE concentrations for 10 min at 37°C. Aggregation was initiated with collagen (190 µg/mL). Aggregation curves were analysed for total percentage aggregation. Values are means ± SEM, n=6. *Statistically significant, $P<0.05$, ** $P<0.01$, compared to the PBS control.

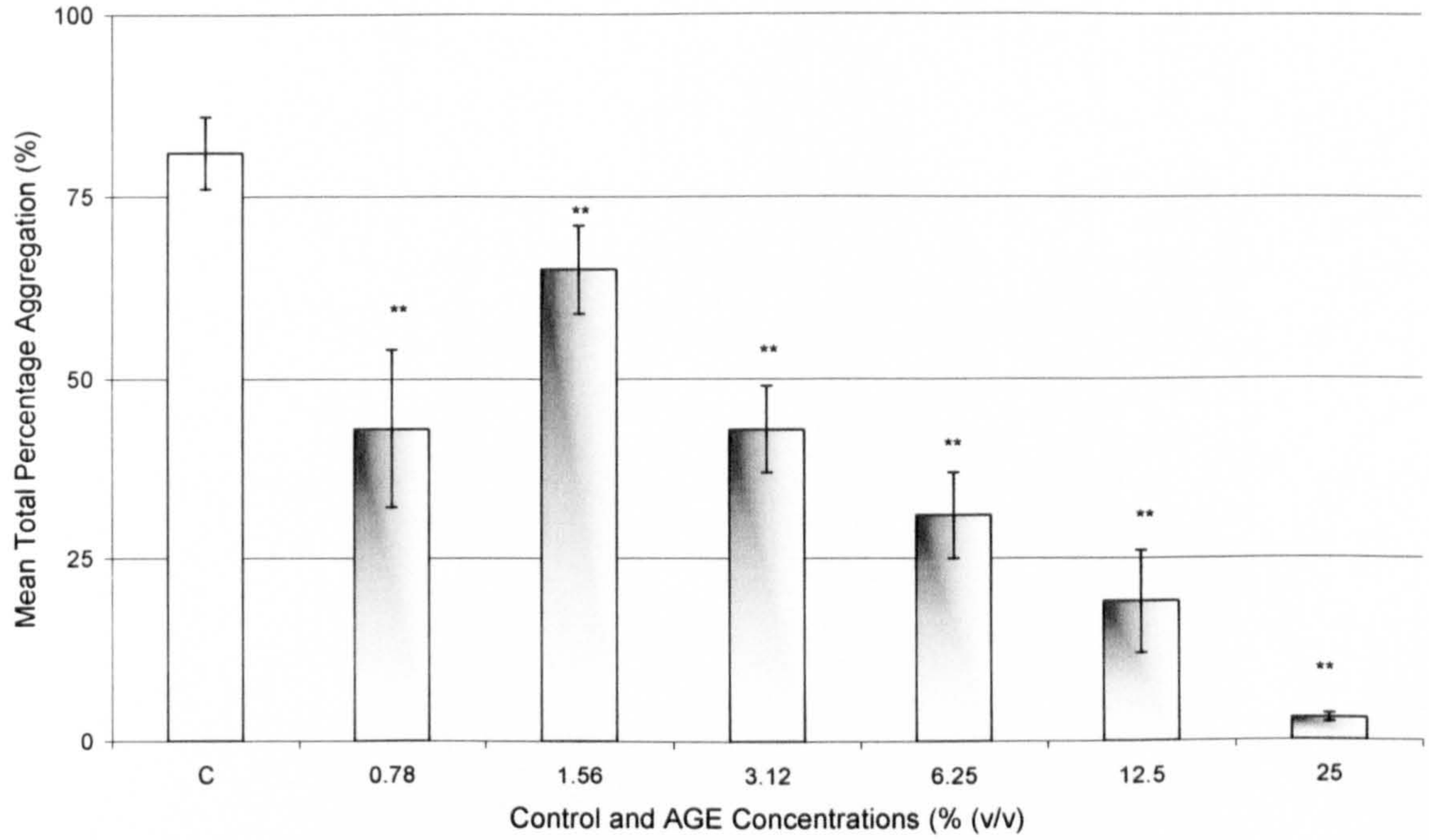


Figure 3.6. AGE and its effect upon thrombin-induced platelet aggregation *in vitro.* PRP was incubated with various AGE concentrations for 10 min at 37°C. Aggregation was initiated with thrombin (0.1 U/L). Aggregation curves were analysed for total percentage aggregation. Values are means ± SEM, n=6. **Statistically significant, $P<0.01$ compared to the PBS control.

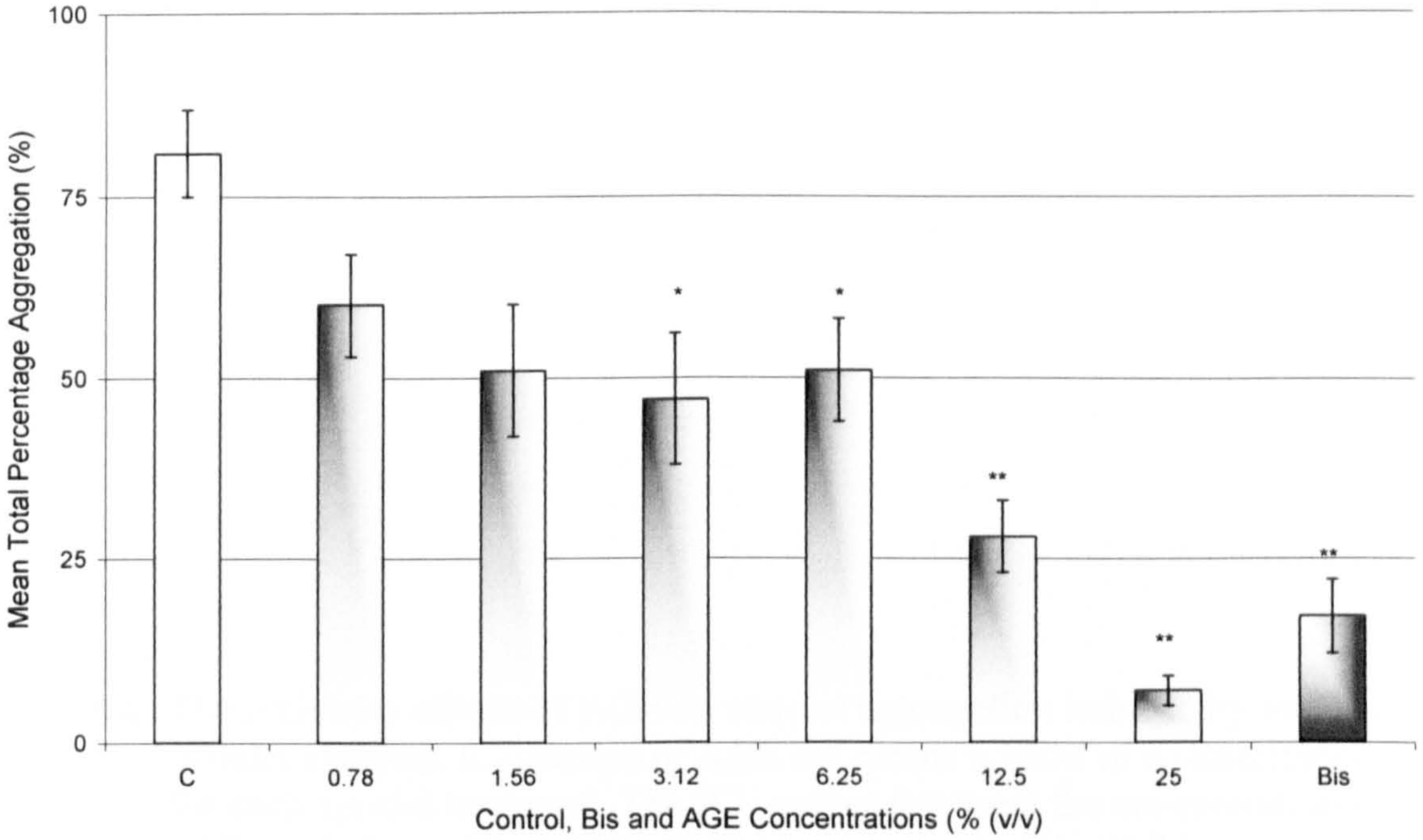


Figure 3.7. AGE and its effect upon PMA-induced platelet aggregation *in vitro*. PRP was incubated with various AGE concentrations for 10 min at 37°C. Aggregation was initiated with PMA (10 μmol/L). Aggregation curves were analysed for total percentage aggregation. Values are means ± SEM, n=6. *Statistically significant, $P<0.05$, **$P<0.01$ compared to the PBS control.

Table 3.2. The inhibitory effects of AGE on platelet aggregation induced by various platelet agonists. IC_{50} values of AGE are means ± SEM of 6 experiments for each agonist treatment. The IC_{50} values represent the concentration of AGE needed to produce 50 % of the maximum possible inhibitory response.

AGONIST	IC_{50} VALUE OF AGE (% v/v)
ADP	3.12 ± 4.0
AA	12.5 ± 2.7
Adrenaline	3.12 ± 7.0
A23187	12.5 ± 4.7
Collagen	6.25 ± 2.6
Thrombin	3.12 ± 2.5
PMA	3.12 ± 3.0

3.2.1.2. Effect of ethanol on ADP-, and collagen-induced platelet aggregation

The purpose of this experiment was to determine whether the enzyme phospholipase D (PLD) plays a significant role in ADP-induced platelet activation and subsequent aggregation, as it is not certain of the exact role if any that this particular enzyme has on platelet aggregation. This was achieved by the indirect examination of the effects of ethanol at different percentage concentrations on both ADP- and collagen-induced platelet aggregation. Ethanol has been shown to inhibit the actions of the PLD and that this enzyme plays an important role within the PLC (phospholipase C) pathway (Houle and Bourgin 1999; Natajaran *et al.* 1996; Nozawa *et al.* 1991) for the generation of phospholipids mediators such as iniositol polyphosphates, diacyclglycerol, and arachidonic acid, all are important in sustaining platelet activation and subsequent platelet aggregate formation. During the manufacture of AGE ethanol is used in the ageing process, and the final product contains ethanol at a concentration of 10 % (v/v). Therefore it is essential to establish that the inhibitory actions of AGE are due to its garlic constituents and not by the non-specific effect of ethanol contained within the AGE product. The recommended daily dose for AGE consumption is 5 mL; the ethanol content contained within this specified dose is less than 0.05 % (v/v), this is the primary reason why concentrations of 0.1-1.0 % (v/v) were used in this study.

Ethanol (0.1-1.0 % v/v) when added to PRP and aggregation initiated with ADP (8 µmol/L), showed no significant inhibition ($P>0.05$) in platelet aggregation when compared to the PBS control (Figure 3.8). Collagen-induced platelet aggregation was dose-dependently inhibited by the addition of ethanol at all the concentrations tested. Significant ($P<0.05$) inhibition of collagen-induced platelet aggregation was noted at the following ethanol concentrations (0.25-1.0 % v/v), when compared to the PBS control (Figure 3.9).

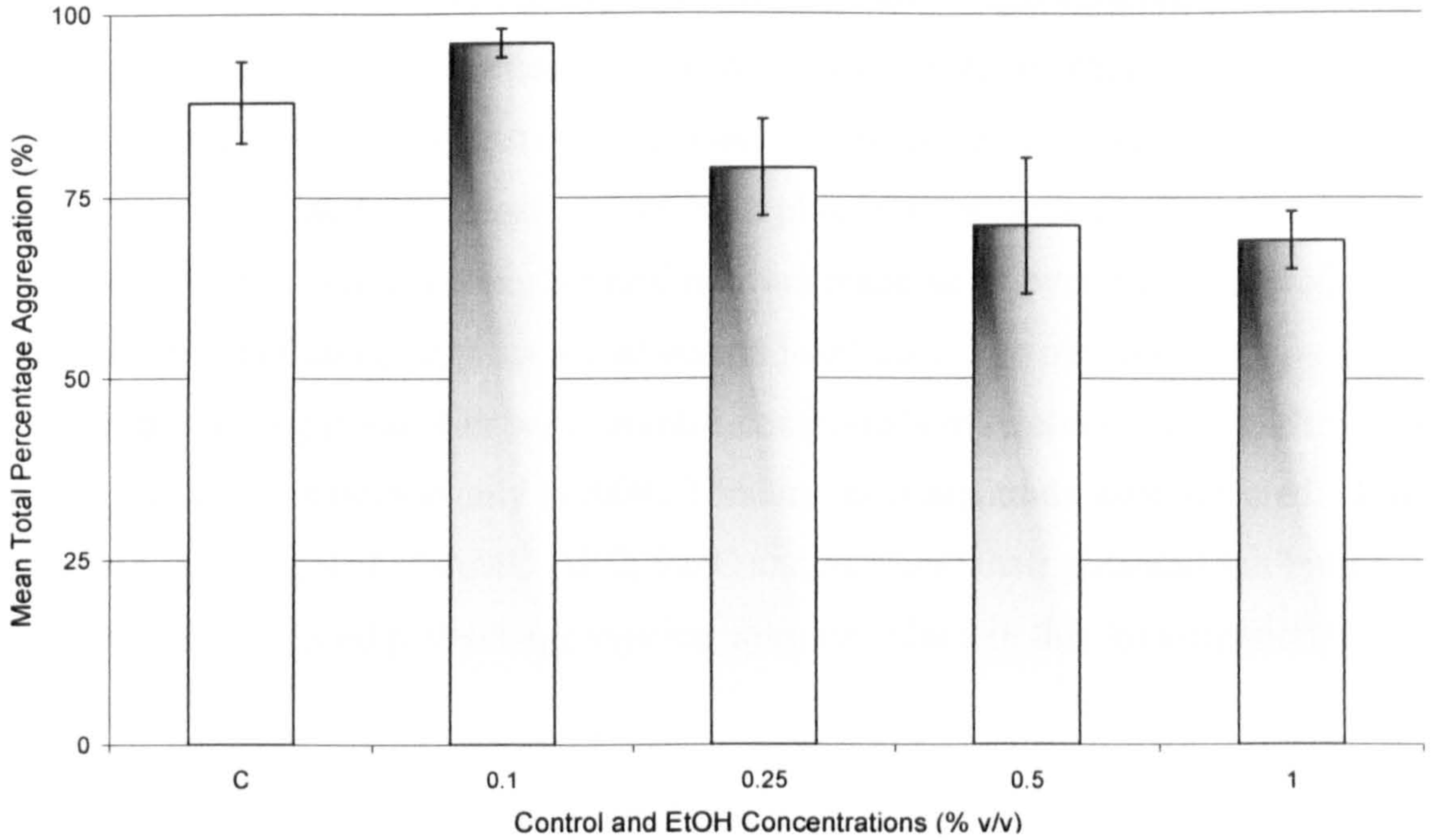


Figure 3.8. The effect of various ethanol concentrations on ADP-induced platelet aggregation. Aggregation curves for ethanol treated platelets were analysed for total percentage aggregation and values are expressed as means ± SEM, n=6. No significant inhibition was detected for any of the ethanol concentrations tested compared to the PBS control.

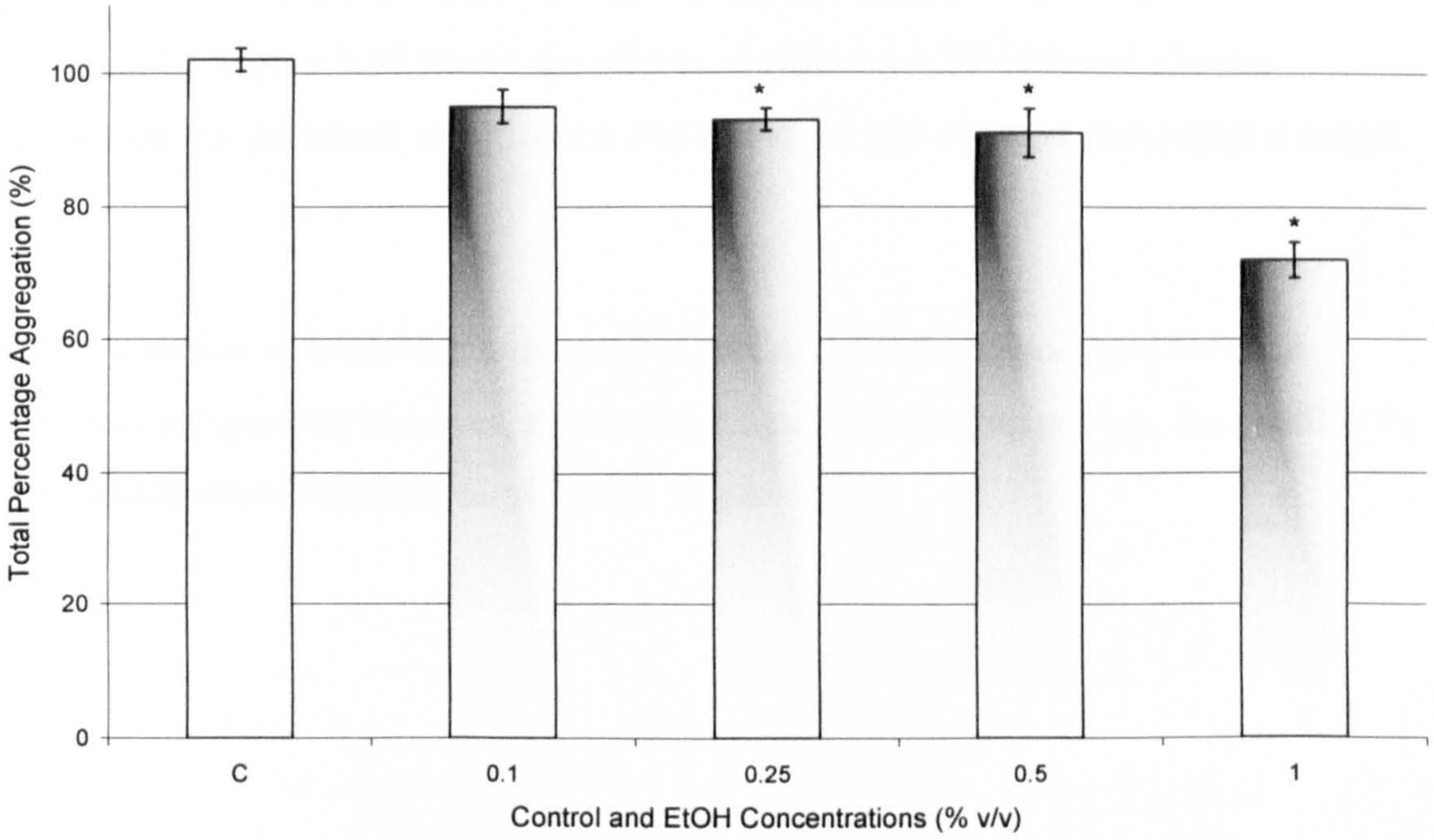


Figure 3.9. The effect of various ethanol concentrations on collagen-induced platelet aggregation. Aggregation curves for ethanol treated platelets were analysed for total percentage aggregation and values are expressed as means ± SEM, n=6. *Statistically significant ($P<0.05$) when compared to the PBS control.

3.2.1.3. Effect of isolated components from AGE on platelet aggregation

It has been established from section 3.2.1 in this study that AGE when pre-incubated with PRP has an inhibitory effect upon agonist-induced platelet aggregation *in vitro*. Ethanol was also tested and found not to inhibit platelet aggregation induced by the agonist ADP. AGE is a complex chemical mixture made up of over 400 plus products which is a result of an aging process that occurs in ethanol. The mixture contains a high concentration of organosulphur water soluble compounds in relation to raw garlic; some of these products are unique only to AGE. Nine initial compounds were selected: alliin, cycloalliin, βC, FA, SAC, SAMC, SEC, SMC and SPC and their potential inhibitory effects on ADP-induced platelet aggregation were examined in this investigation (0.78 – 100 μmol/L).

All the components when tested follow a similar biphasic pattern in the way they affect ADP-induced platelet aggregation. The results obtained showed that none of the individual components are very effective in inhibiting ADP-induced platelet aggregation in relation to AGE as a whole compound (data not shown, refer to Appendix V). Figure 3.10 shows the effects of alliin on ADP-induced platelet aggregation, no statistical significance was found for any of the concentrations tested.

NB. A reduction in inhibition was noted at various concentrations used for each individual component, however, the results did not follow a set pattern, the profiles for each constituent of AGE are displayed in Appendix V.

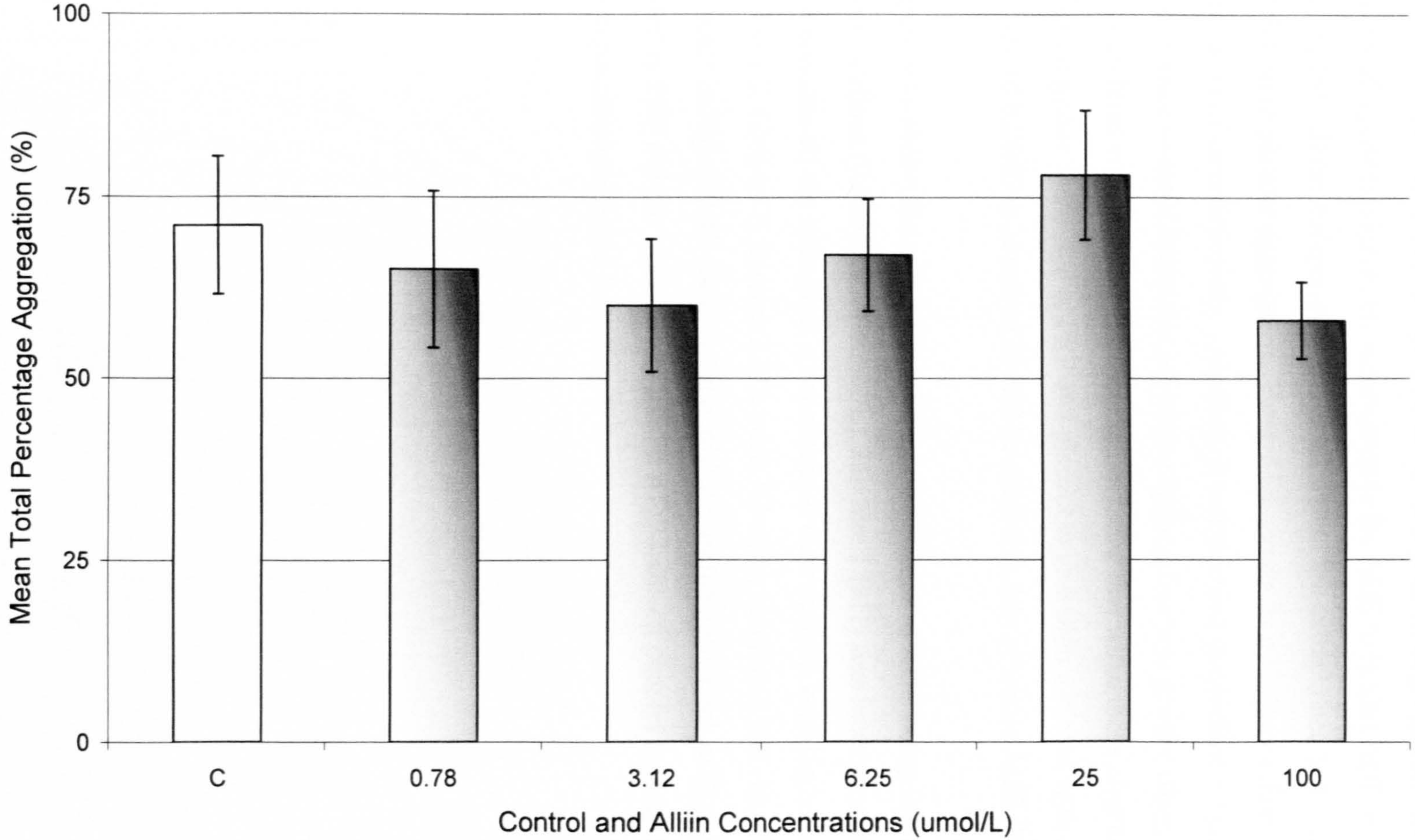


Figure 3.10. The effect of various Alliin concentrations on ADP-induced platelet aggregation. Aggregation curves were analysed for total percentage aggregation and values are expressed as means ± SEM, n=6. No statistical difference between alliin concentrations and PBS control (P value>0.05).

This study has shown that the individual components of AGE are not effective individually as inhibitors of ADP-induced platelet aggregation. It has been found that AGE as a whole extract can significantly inhibit platelet aggregation induced by a variety of agonists. Hence, the assumption is for AGE to inhibit ADP-induced platelet aggregation all the components contained within the extract need to be present for inhibition of platelet aggregation. Thus, AGE exerts its inhibitory effect through synergism of its constituents. Therefore, it was decided to combine all nine components as a mixture to see if inhibition of platelet aggregation was greater when compared to the individual components. The assumption being that when these nine components are mixed together and added to PRP and platelet aggregation initiated with ADP, the amount of inhibition observed should be similar to that of whole AGE (Figure 3.1)

Figure 3.11 shows the effect of a mixture of the isolated AGE components at different concentrations (0.000195-1.0 mmol/L). Mixture 1 (M1) was incorporated into this study as it contains all the individual components at their optimal working concentrations (Table 2.3, Chapter 2, Section 2.3.2, page 36) when examined individually. The results depicted in Figure 3.12 show that the mixtures of the individual AGE components when added to PRP did not significantly inhibit ADP-induced platelet aggregation at any of the concentrations tested including M1.

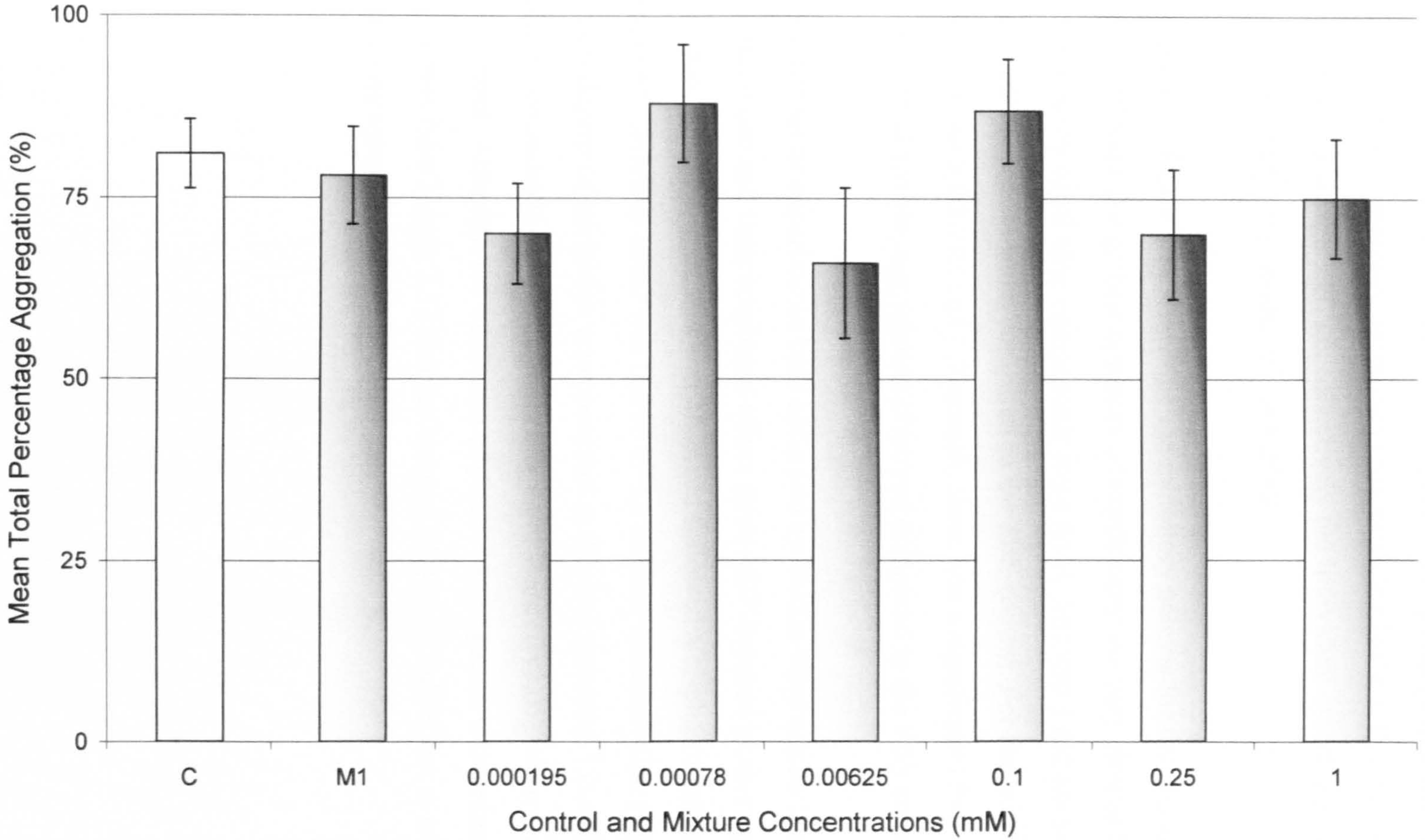


Figure 3.11. The effect of a mixture of the isolated AGE components (Alliin, Cycloalliin, βC, FA, SAC, SAMC, SEC, SMC and SPC) on ADP-induced platelet aggregation. M1 – mixture 1 contains all nine components at concentrations that produced optimal inhibition of platelet aggregation when tested individually. The remaining concentrations contain all nine components at specific concentrations (0.00019-1.0 mmol/L). Aggregation curves were analysed for mean total percentage aggregation. Values are expressed as means ± SEM, n=6. No statistical significance was calculated ($P>0.05$)

A mixture of cysteine sulphoxides (SAC, SAMC, SEC, SMC and SPC) was compared to the inhibitory profile of AGE (Figure 3.1). All the members of this particular cysteine family have very similar chemical structures and physical properties i.e. all are water soluble, sulphur-containing amino acid compounds (Table 3.1), and should theoretically behave in a similar manner. i.e. collectively the individual cysteine sulphoxides should enhance the inhibitory actions of each other.

The results in figure 3.12 show that the inhibitory actions for the mixture of all five cysteine sulphoxides at their optimum concentrations are very similar to that obtained for the mixture of all nine components from AGE. Mixture 1 did not result in any significant difference when compared to the PBS control. For the mixture concentrations 0.000195 and 1.0 mmol/L there was a significant ($P<0.05$) reduction in the amount of platelet aggregation observed compared to the PBS control..

In another set of experiments both alliin and cycloallin were mixed together at various concentrations and their combined effect upon ADP-induced platelet aggregation was examined. The inhibition that was observed followed a bi-phasic pattern in that the amount of inhibition noted was linked to the mixture concentration (Figure 3.13). The greatest degree of inhibition was prevalent at the concentration 1.0 mmol/L. With all the mixture concentrations being statistically significant ($P<0.05$) when compared to the PBS control. Although the inhibition of platelet aggregation was deemed significant, it is apparent from Figure 3.13 that the amount of platelet inhibition is not comparable to AGE in its natural form.

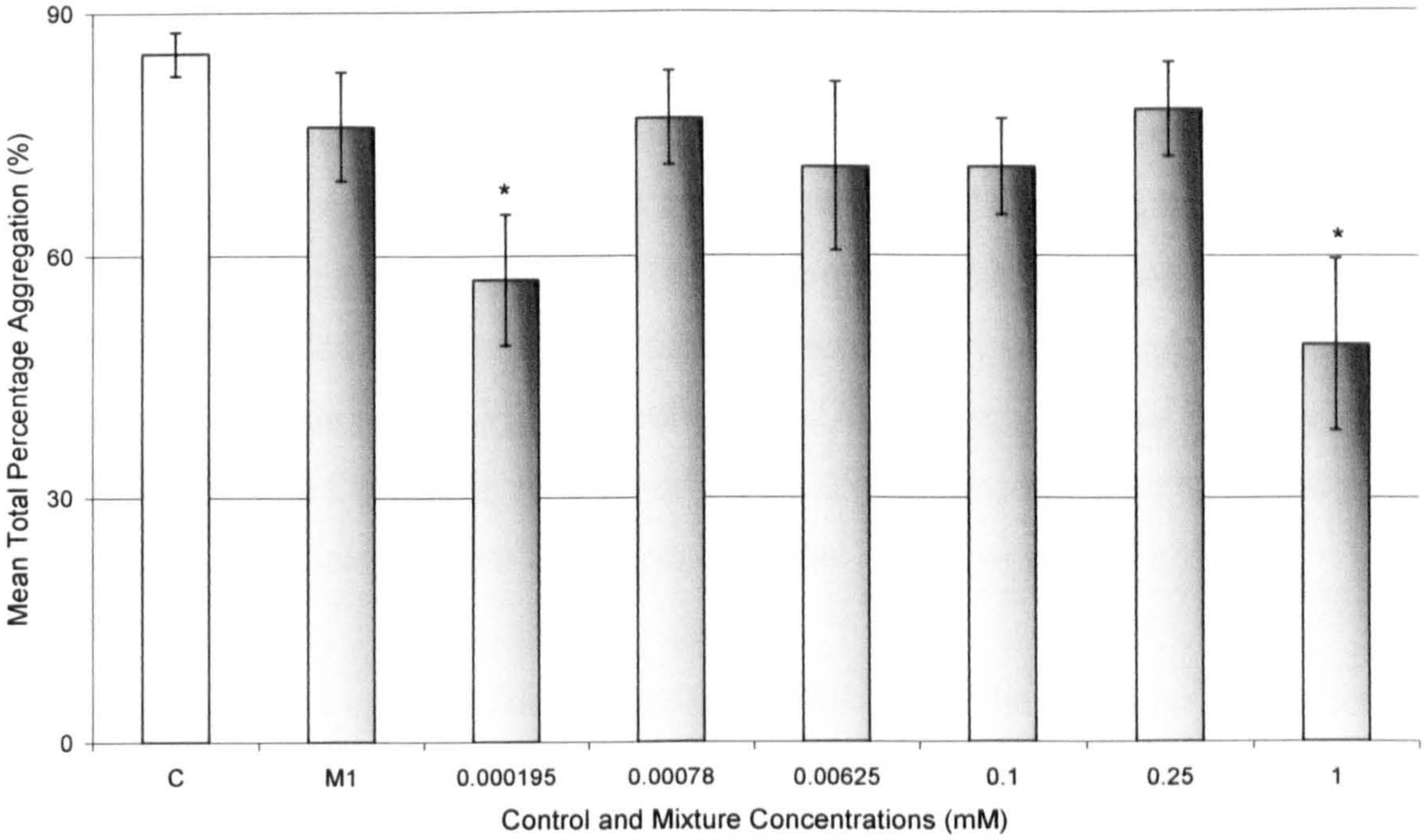


Figure 3.12. The effect of a cysteine sulphoxide mixture on ADP-induced platelet aggregation SAC, SAMC, SEC, SMC and SPC). M1 – mixture 1 contains all five components at concentrations that produced optimal inhibition of platelet aggregation when tested individually (Refer to Table 2.3, page 36). The remaining concentrations contain all nine components at specific concentrations (0.00019-1.0 mmol/L). Aggregation curves were analysed for mean total percentage aggregation. Values are expressed as means ± SEM, n=6. *Statistically significant when compared to the PBS control.

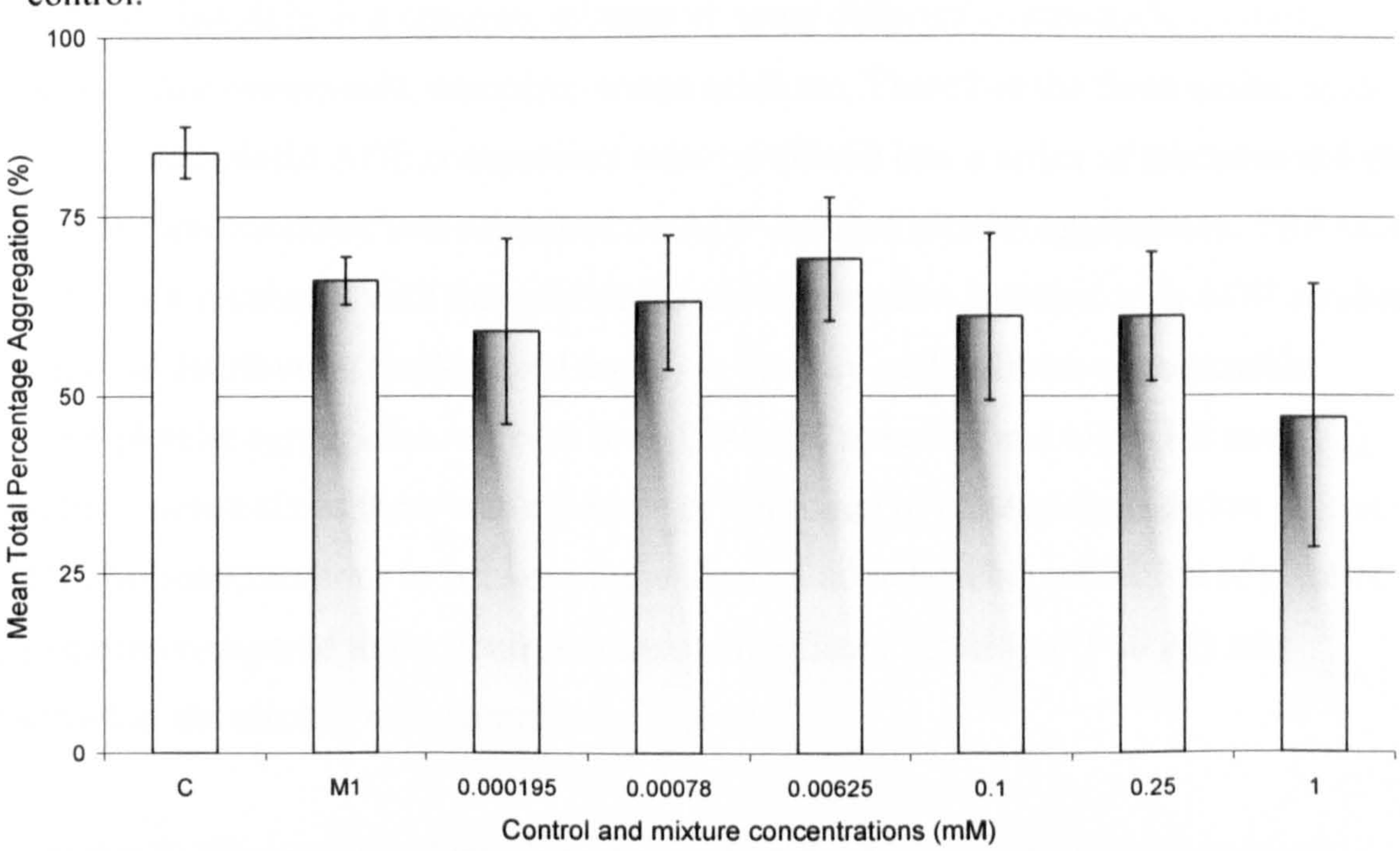


Figure 3.13. The effect of an alliin and cycloalliin mixture on ADP-induced platelet aggregation. M1 – mixture 1 contains both components at concentrations that produced optimal inhibition of platelet aggregation when tested individually. The remaining mixtures contain both components at specific concentrations (0.00019-1.0 mmol/L). Aggregation curves were analysed for mean total percentage aggregation. Values are expressed as means ± SEM, n=6. All the mixture concentrations are statistically significant ($P<0.05$) when compared to the PBS control.

3.2.1.4. The effect of amino acids found in AGE on ADP-induced platelet aggregation

The amino acids L-arginine, L-cysteine and L-methionine were not efficient at inhibiting ADP-induced platelet aggregation. Figure 3.14 shows that L-methionine was the only amino acid significantly effective in inhibiting ADP-induced platelet aggregation at the concentration range 0.001-1.0 mmol/L. The exception being at 0.006 mmol/L, at this particular concentration the mean total percentage aggregation value was very similar to that of the PBS control sample. At the concentration 9.0 mmol/L, both L-arginine and L-cysteine significantly ($P<0.05$) inhibited platelet aggregation when compared to their respective controls, whereas L-methionine did not.

A mixture of all three amino acids was added to PRP before the initiation of platelet aggregation with ADP. It was found that as a mixture the amino acids were not efficient at reducing the amount of platelet aggregation observed compared to the PBS control. Inhibition was observed at the mixture concentration 0.00078 mmol/L (Figure 3.15). The results were not statistically significant ($P>0.05$) when compared to the PBS control.

It is known that AGE is a complex mixture of many different compounds, including organosulphur compounds, saponins, amino acids etc. Therefore the three amino acids plus the nine isolated AGE components were combined into a series of mixtures and the effects of these mixtures was examined on ADP-induced platelet aggregation. PRP that had been pre-incubated with these mixtures and aggregation initiated with ADP resulted in a normal distribution (bell-shaped curve) in the way each mixture concentration affected platelet aggregation. At both low (0.000195 mmol/L) and high (1.0 mmol/L) mixture concentrations there was evidence of inhibition of platelet aggregation (Figure 3.16). The concentrations in between these showed no noticeable inhibition of platelet aggregation compared to the control sample. Significant inhibition ($P<0.05$) was observed at the mixture concentration 1.0 mmol/L.

The major finding generated in this section is that for AGE to inhibit ADP-induced platelet aggregation, AGE in its natural form is required to bring about the desired effects. Hence, synergism of all AGE components must be taking place for inhibition to occur effectively.

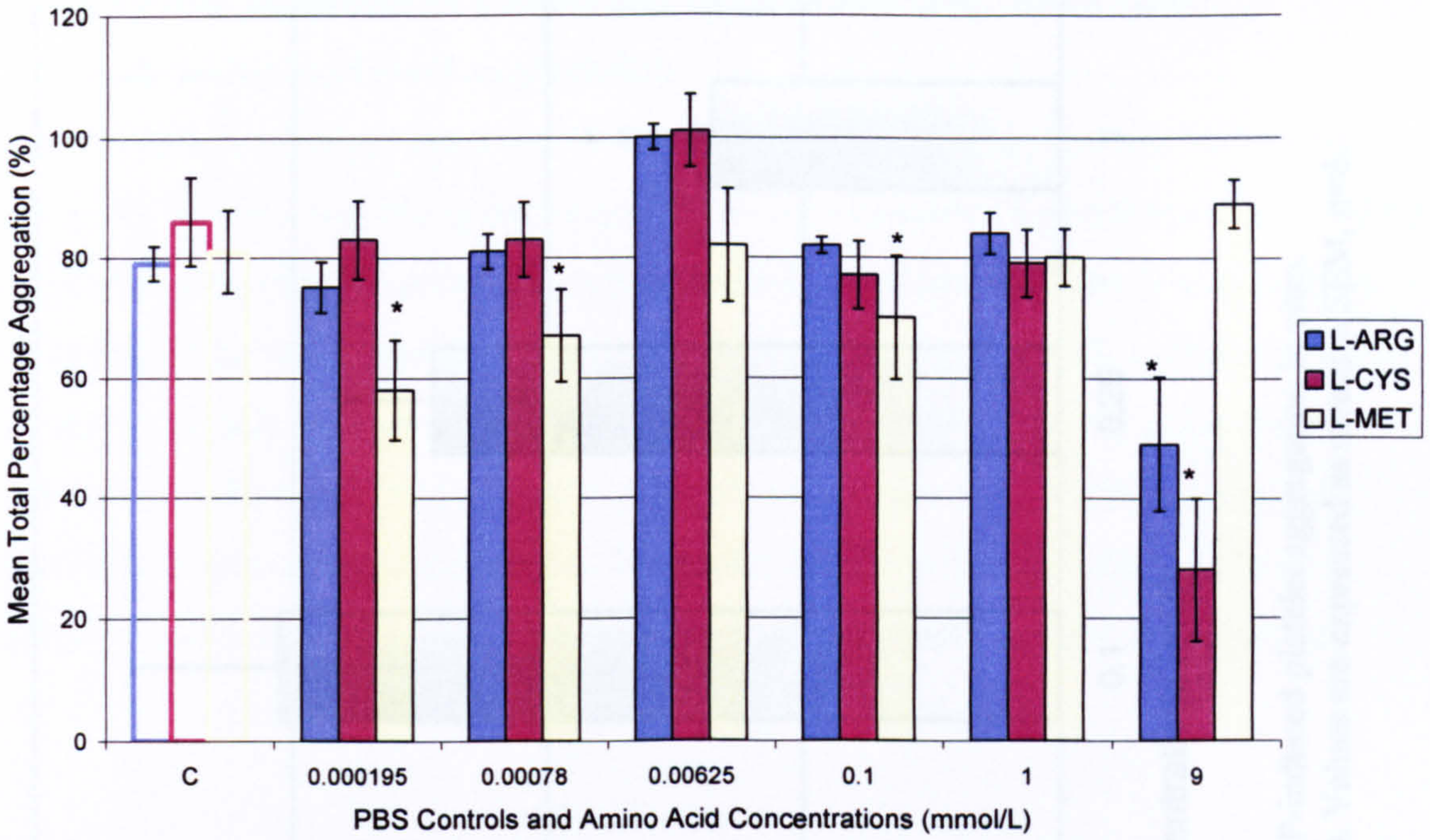


Figure 3.14. The effect of individual amino acids on ADP-induced platelet aggregation (L-arginine, L-cysteine and L-methionine). Aggregation curves were analysed for mean total percentage aggregation and the values are expressed as means ± SEM, n=6. *Statistically significant ($P<0.05$) compared to the PBS control.

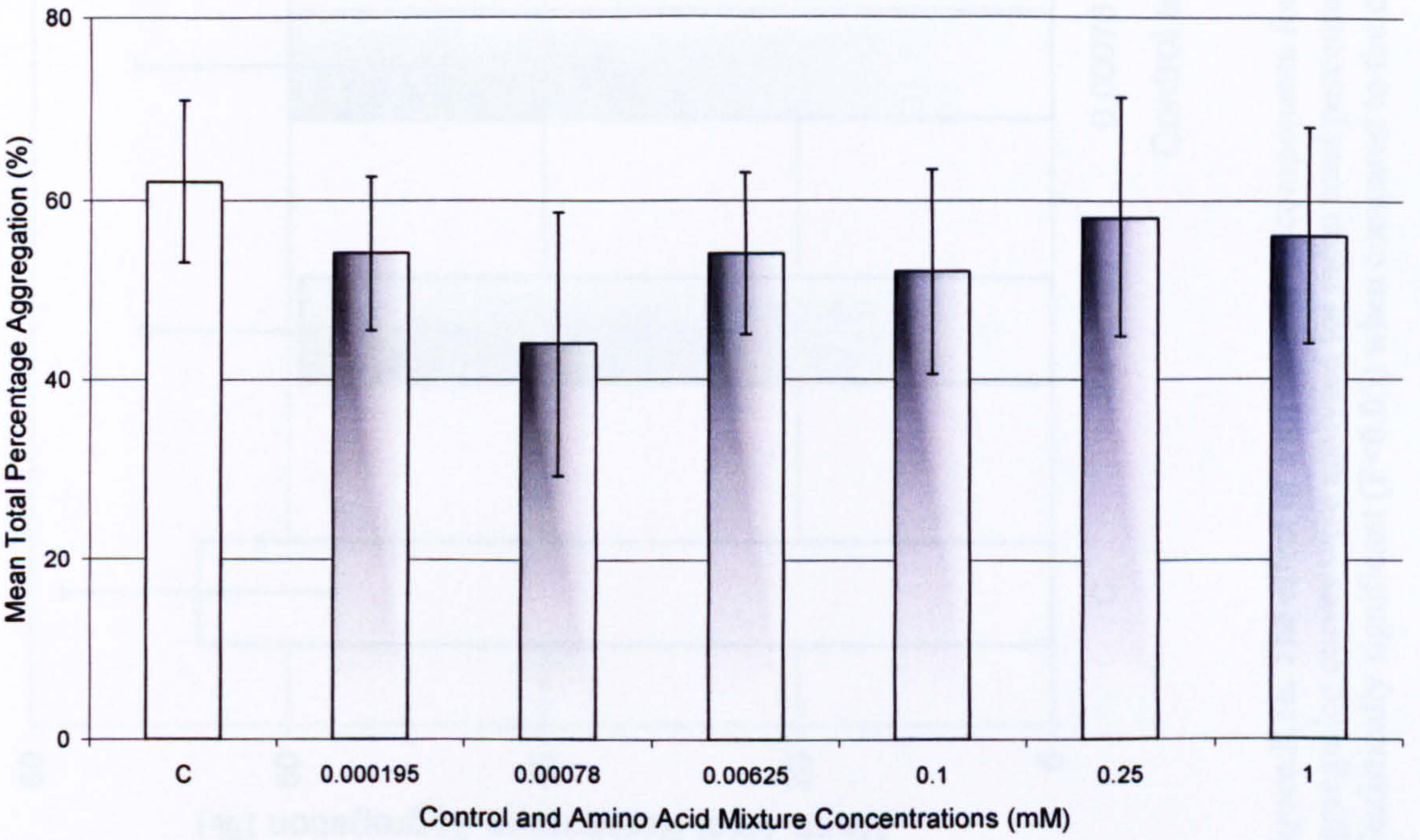


Figure 3.15. The effect of an amino acid mixture on ADP-induced platelet aggregation. Aggregation curves were analysed for mean total percentage aggregation and values are expressed as means ± SEM, n=6. There was no statistical significance ($P>0.05$) noted for any of the mixture concentrations when compared to the PBS control.

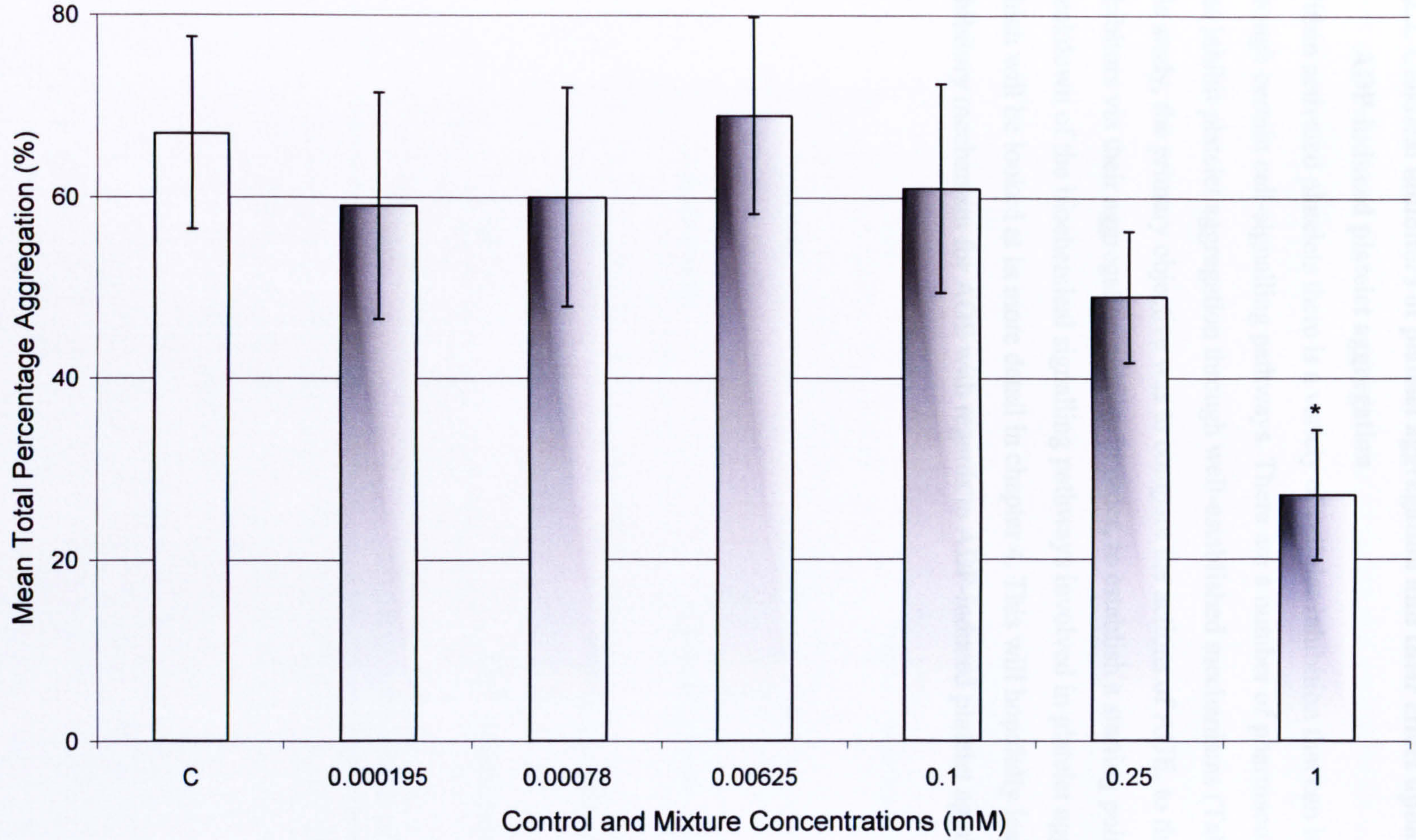


Figure 3.16. The effect of a mixture of 12 components found in AGE on ADP-induced platelet aggregation in vitro. Aggregation curves were analysed for mean total percentage aggregation (%). Values are expressed as means ± SEM, n=6. *Statistically significant ($P<0.05$) when compared to the control.

3.2.2. Classical inhibitors of platelet aggregation and their effect upon ADP-induced platelet aggregation

Within activated platelets there is a variety of cellular inhibition that can be targeted through certain cell-signalling pathways. There are a number of pharmacological agents that inhibit platelet aggregation through well-established mechanisms (Table 3.3). In this study, the primary objective was to compare the actions of AGE, to that, of known inhibitors via their aggregation profiles in order, to establish a starting point for the breakdown of the biochemical signalling pathways involved in platelet aggregation which will be looked at in more detail in chapter 4. This will hopefully lead to an inhibitory mechanism for AGE with regards to ADP-induced platelet aggregation.

Table 3.3. Classical inhibitors of platelet aggregation and their mechanism of inhibition.

Classical Inhibitor	Mechanism of Inhibition
AlF_3	G-proteins
AG527 - Tyrphostin	Protein tyrosine kinases
BIS	Protein kinase C
GEN	Protein tyrosine kinases
IBMX	cAMP and cGMP phosphodiesterases
Indomethacin	Cyclooxygenase inhibitor
OKA	Protein phosphatases
NaF	Ca^{2+} mobilisation
PGE_1	Increases intracellular cAMP levels

The inhibitors of platelet aggregation listed in table 2.5 (Chapter 2, Section 2.4.3, page 38) were incubated with PRP and platelet aggregation was initiated with ADP and aggregation traces results were compared to that of AGE (25 % v/v). The mechanistic actions of all these inhibitors are well documented in the literature (Dhar 1993; Feijge *et al.* 2004; Ferri *et al.* 1994; Gazit *et al.* 1989; Kikura *et al.* 2000; Kondo *et al.* 1991; Kong 2004; Liu *et al.* 1998; Schwarz *et al.* 2001).

The first classical inhibitor that was tested was aspirin which is used clinically in the treatment of CVD. Its mechanistic action is through the inhibition of platelet aggregation is by interfering with AA metabolism via the suppression of the enzyme cyclooxygenase. Thus, prostaglandins that aid the aggregation process are unable to be formed. Aspirin inhibited ADP-induced platelet aggregation at the following concentrations: 0.00078, 0.1 and 0.5 mmol/L. The effect of aspirin was not dose-dependent, and statistically significant inhibition ($P<0.05$) was only observed at 0.00078 mmol/L (Figure 3.17); the aspirin concentration 0.1 mmol/L reduced platelet aggregation compared with the other concentrations used but was not deemed significant following statistical analysis.

PGE_1 was dissolved in DMSO (concentration did not exceed 0.05 %, v/v in PRP sample) and diluted to its final concentrations in PBS. The following final concentrations (7.5, 15, 31.25, 62.5, 125 and 250 µg/mL) were pre-incubated with PRP prior to the initiation of platelet aggregation with ADP. As expected, PGE_1 at all the concentrations tested significantly ($P<0.01$) inhibited ADP-induced platelet aggregation when compared to the control (Figure 3.18).

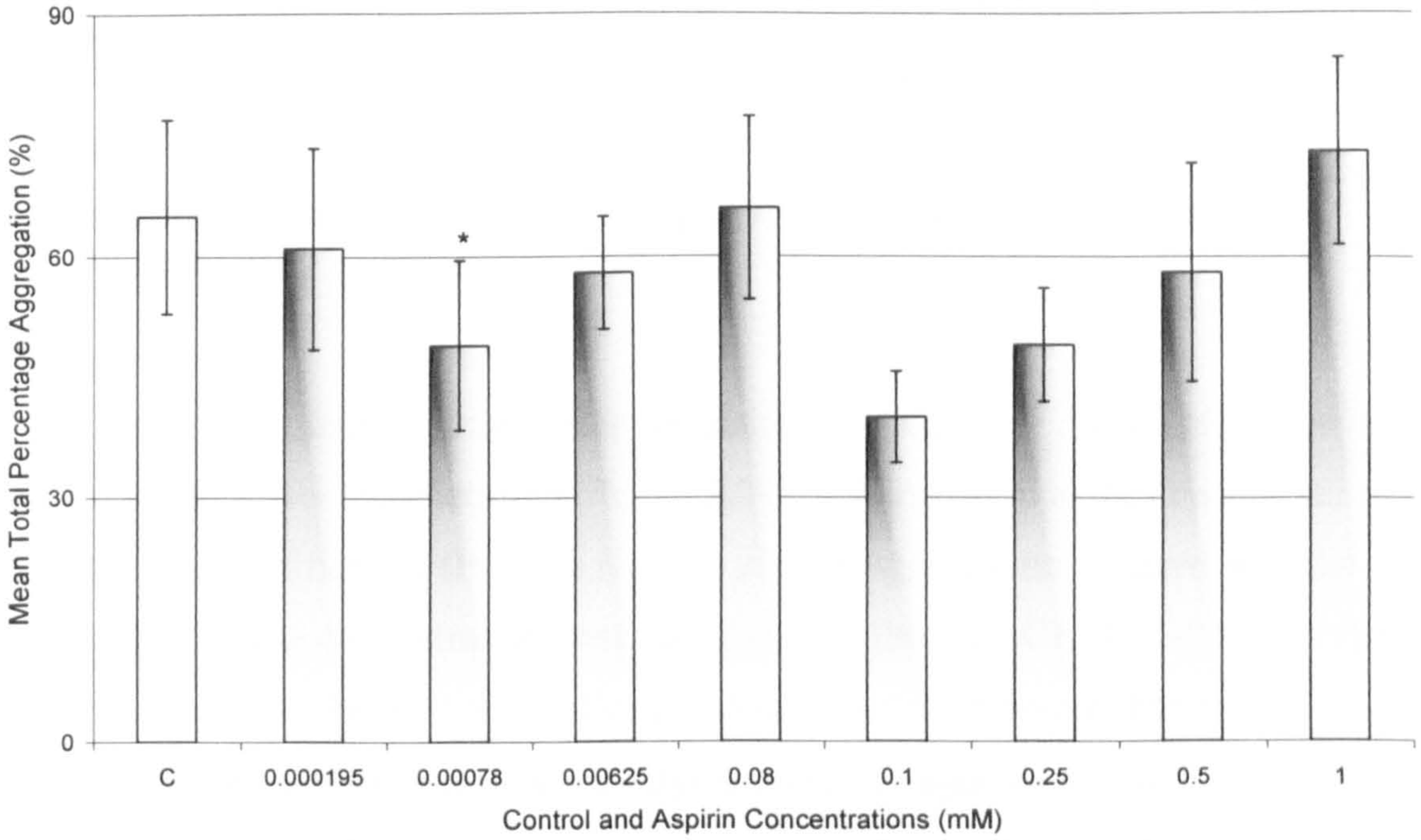


Figure 3.17. The effect of various aspirin concentrations on ADP-induced platelet aggregation. Aspirin (0.00078-1.0 mmol/L) was added to PRP for 10 min at 37°C, aggregation was initiated via the addition of ADP. Aggregation curves were analysed for mean total percentage aggregation. Values are expressed as means ± SEM, n=6.
*Statistically significant ($P<0.05$) compared to the control.

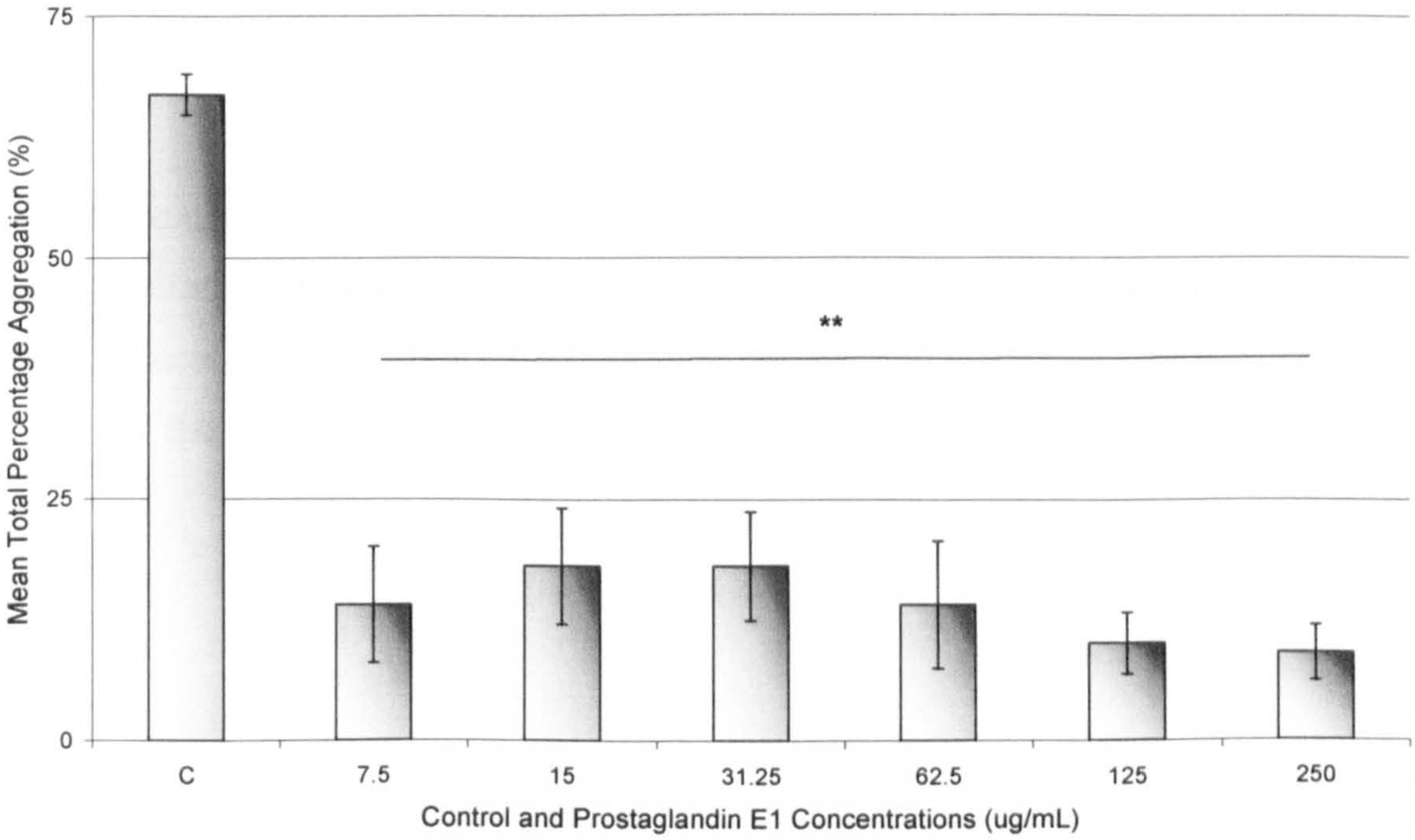


Figure 3.18. The effect of various PGE_1 concentrations on ADP-induced platelet aggregation. PGE_1 (7.5-250 µg/mL) was added to PRP for 10 min at 37°C, aggregation was initiated via the addition of ADP. Aggregation curves were analysed for mean total percentage aggregation. Values are expressed as means ± SEM, n=6.
**Statistically significant ($P<0.01$) compared to the control.

Seven more classical inhibitors (AlF_3, AG527, BIS, GEN, IBMX, NaF and OKA) of platelet aggregation were examined and their aggregation profiles were compared to that of AGE (Figure 3.19). The inhibitors GEN and IBMX were the only ones that significantly ($P<0.01$) inhibited platelet aggregation induced by the agonist ADP.

In another set of experiments the inhibitory actions of AGE were compared to that of the classical inhibitor indomethacin (Figure 3.20). Indomethacin at the concentrations 0.015-1.0 mM significantly ($P<0.01$) reduced platelet aggregation initiated with the agonist ADP. These concentrations were compared to that of AGE (12.5-25 %, v/v) and it was observed that the inhibitory actions of AGE were more potent than that of indomethacin on ADP-induced platelet aggregation. Collagen was used to ensure that indomethacin was working correctly, as its mode of action is through the suppression of the cyclooxygenase pathway; and this pathway is involved in collagen-induced platelet aggregation. Indomethacin treated platelets when challenged with collagen resulted in significant inhibition of platelet aggregation. Indomethacin had a more profound inhibitory effect upon collagen-induced platelet aggregation than during platelet aggregation initiated with the agonist ADP.

Following a comparison to AGE the classical inhibitors PGE_1, GEN, IBMX and indomethacin had similar inhibitory aggregation profiles to that of AGE. The pathways that are affected by these four inhibitors were investigated further and the results are reported in the in the next chapter.

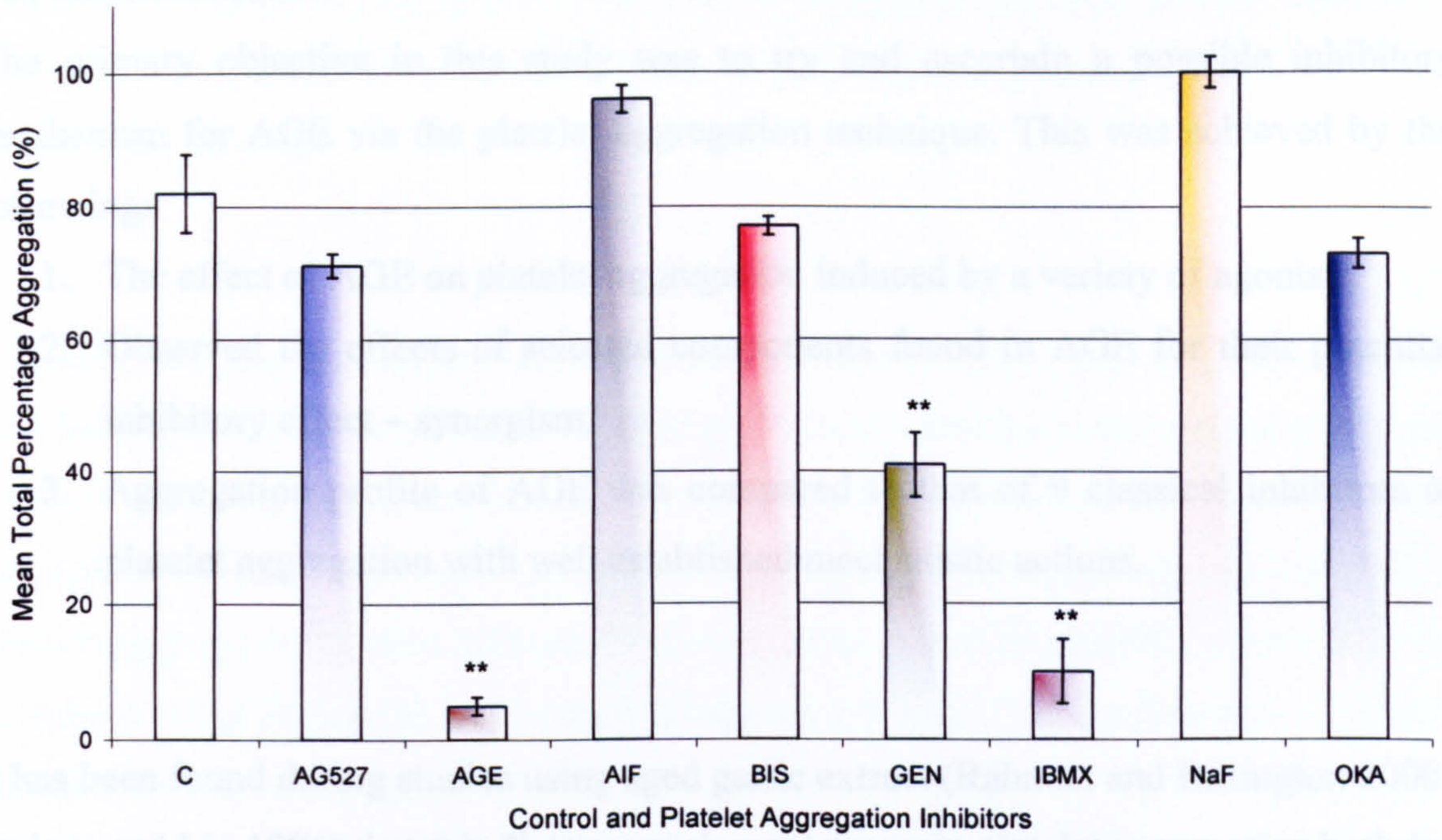


Figure 3.19. The effect of seven known platelet aggregation inhibitors compared to AGE upon ADP-induced platelet aggregation. AlF_3 (100 µmol/L), AG527 (25 µmol/L), BIS (100 µmol/L), GEN (100 µmol/L), IBMX (150 µmol/L), NaF (100 µmol/L) and OKA (4 µmol/L) were incubated with PRP and aggregation was initiated with ADP (8 µmol/L). The aggregation curves were analysed for mean total percentage aggregation and the results were compared to that of AGE (25 % v/v). Values are expressed as means ± SEM, n=6. **Statistically significant ($P<0.01$) when compared to the PBS control.

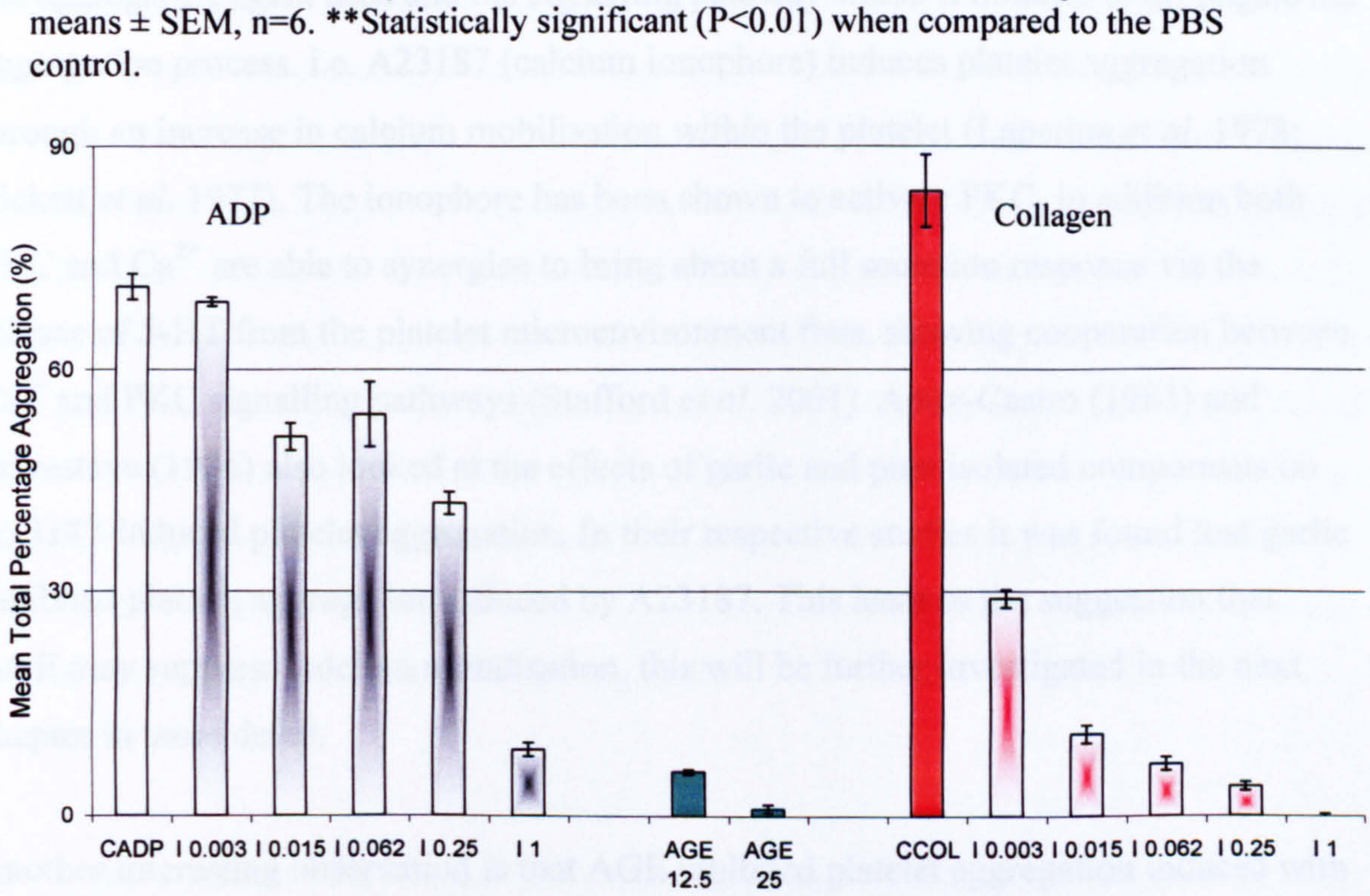


Figure 3.20. The effect of indomethacin on ADP- and collagen-induced platelet aggregation. PRP was incubated with either indomethacin (0.003-1.0 mM) or AGE (12.5-25 %, v/v) for 10 min at 37°C. Platelet aggregation was initiated with ADP (8 µmol/L) or collagen (190 µg/mL). AGE treated samples were activated with ADP. Values are expressed as means ± SEM, n=6. All the concentrations (AGE and indomethacin) are statistically significant ($P<0.01$) when compared to the controls. Exception indomethacin concentration 0.003 mM when aggregation was stimulated with ADP.

3.3. DISCUSSION

The primary objective in this study was to try and ascertain a possible inhibitory mechanism for AGE via the platelet aggregation technique. This was achieved by the following:

1. The effect of AGE on platelet aggregation induced by a variety of agonists.
2. Observed the effects of selected components found in AGE for their potential inhibitory effect – synergism.
3. Aggregation profile of AGE was compared to that of 9 classical inhibitors of platelet aggregation with well established mechanistic actions.

It has been found during studies using aged garlic extract (Rahman and Billington 2000 Steiner and Lin 1996) that this dietary supplement prevents platelet aggregation both *in vitro* and *in vivo.* In this study it was found that the pre-treatment of human platelet-rich plasma with various AGE concentrations resulted in inhibition of platelet aggregation for all the agonists examined. The degree of inhibition observed was directly related to the aggregating agent used and the signalling pathway which it initiates to propagate the aggregation process. i.e. A23187 (calcium ionophore) induces platelet aggregation through an increase in calcium mobilisation within the platelet (Lapetina *et al.* 1978; Pickett *et al.* 1977). The ionophore has been shown to activate PKC, in addition both PKC and Ca^{2+} are able to synergise to bring about a full secretion response via the release of 5-HT from the platelet microenvironment thus, showing cooperation between Ca^{2+} and PKC signalling pathways (Stafford *et al.* 2001). Apitz-Castro (1983) and Srivastava (1986) also looked at the effects of garlic and pure isolated components on A23187-induced platelet aggregation. In their respective studies it was found that garlic inhibited platelet aggregation induced by A23187. This leads to the suggestion that AGE may suppress calcium mobilisation, this will be further investigated in the next chapter in more detail.

Another interesting observation is that AGE inhibited platelet aggregation induced with the agonist PMA. It is known that PMA directly activates the enzyme PKC and, that this enzyme is involved in cytoskeleton rearrangement and the release of granule contents such as ADP and 5-HT into the platelet extracellular environment which further propagates platelet aggregation. This enzyme is common to all forms of agonist

stimulation of platelet aggregation. Therefore, it is possible that AGE directly inhibits PKC activity or inhibits a point downstream of PKC signalling during the platelet aggregation process.

The major finding in this study was that AGE at concentrations >6.25 % (v/v) inhibited platelet aggregation. These results validate previous studies that examined the effects of AGE on platelet aggregation (Steiner and Lin 1998; Steiner and Li 2001; Rahman and Billington 2000). Thus, it can be concluded that AGE directly affects agonist-induced platelet aggregation *in vitro*. This study also suggests that AGE possibily exerts its inhibitory effect during the latter stages of platelet activation/aggregation and not at the receptor level for each agonist.

It has been suggested in the literature that the weak agonists such as ADP activate platelets via the phospholipase A (PLA) pathway in the early stages of agonist-induced platelet aggregation (Kunapuli 1998; Puri 1999). During the secondary phase of activation with the release of the dense granule contents the phospholipase C (PLC) pathway is brought into play (Brass *et al.* 1993). This is not the scenario for strong agonists such as collagen, as they activate the PLC pathway directly upon collagen binding to its receptor on the platelet cell surface (Packham and Mustard 2005).
This was not reflected in the results obtained during this study as ADP-induced platelet aggregation was inhibited by the inhibitors GEN, IBMX indomethacin and PGE_1. All of which are common to all forms of agonist stimulation. Hence, AGE may not exert its effect during the primary stages of platelet activation but rather during the metabolism and release of second messengers and possibly the release of dense granule contents and the bioavailability of calcium within the platelet.

The main purpose of this study was to determine the inhibitory mechanisms of AGE within the platelet. In order to achieve this goal, ADP was chosen as the agonist of choice, its biochemical pathway has been well established over recent years within the platelet. Before the pathway was dissected it was imperative to establish that the inhibitory responses observed by AGE was due to the natural constituents present

within AGE and not by the non-specific effects of products such as ethanol that are used during the aging process and are present within the final product.

Ethanol is used during the manufacture of AGE; therefore it is essential that the inhibition of platelet aggregation observed with AGE is due to the garlic constituents and not the ethanol. This was done indirectly by examining the effects of ethanol at different concentrations on ADP-induced platelet aggregation. As the enzyme phospholipase D (PLD) activity is retarded by ethanol and this enzyme plays an important role within the PLC pathway (Houle and Bourgin 1999; Natajaran *et al.* 1996; Nozawa *et al.* 1991). It was found in this study that ethanol did not significantly inhibit ADP-induced platelet aggregation (Figure 3.8) compared to the PBS control. These results are similar to those obtained by de Lange *et al.*, (2003) in that they too found that ethanol present in red wine did not inhibit platelet aggregation and the observed inhibition was a result of polyphenols present within the red wine. Figure 3.8 shows that there is the possibility that at a much higher concentration of ethanol ADP-induced aggregation may be inhibited. Although this may be the case and further investigation would be required, it is highly unlikely that the daily recommended dose of AGE (5 mL/day) will contain more than 1 % ethanol. Therefore, ethanol does not inhibit ADP-induced platelet aggregation and the inhibition that was observed following AGE incubation with platelets is due solely to other constituents and not any ethanol present within the liquid AGE extract.

The literature states that phosphatidic acid (PA) production and secretion is inhibited by ethanol and that ethanol also inhibits arachidonate release (Houle and Bourgin., 1999; Huang *et al.* 1991). If this is the case, then why was there no inhibition of aggregation stimulated by ADP? The answer to this question is that not all agonists stimulate activation and subsequent aggregation to the same degree. This is due to the strength of the agonist used in the initiation of aggregation. ADP, a weak agonist on its own cannot stimulate PLD activity, but can reinforce the effects of low doses of stimulatory agents such as thrombin and collagen (Exton 1999; Frohman and Morris 1999). Thus, ADP initially activates the PLA_2 signalling pathway upon platelet activation. Hence, this may be a reason why ethanol did not inhibit ADP-induced aggregation.

To test the theory that strong agonists activate PLC signalling mechanisms directly upon platelet activation collagen was used as a replacement for ADP. Ethanol significantly inhibited collagen-induced platelet aggregation (Figure 3.9). These results aid in the study of the mechanisms by which AGE inhibits platelet aggregation. It can be assumed that ADP induces a different biochemical pathway during activation to that of collagen. PLC is possibly still activated during ADP-induced aggregation but is not an essential component.

AGE is a complex chemical mixture which is rich in water-soluble organosulphur compounds. Although some of these compounds are found in raw garlic, their content within AGE is increased considerably during the aging process and unique products such as SAC, and FA are formed during aging and extraction which are unique only to AGE (they are not found in either raw or heat-treated garlic).

The following twelve components found within AGE were examined: alliin, beta-chlorogenin, cycloalliin, and fructosylarginine, L-arginine, L-cysteine, L-methionine, S-allylcysteine, S-allylmercaptocysteine, S-ethylcysteine, S-methylcysteine and S-propenylcysteine. It was found that each individual component was not effective at inhibiting platelet aggregation both on its own and as a mixture. The components were not as effective as AGE in its natural form in inhibiting platelet aggregation. This leads to the conclusion that the twelve components of AGE act synergistically along with many other compounds present in AGE to bring about this observed inhibitory effect, as these isolated components are not the only inhibitors of platelet aggregation found within AGE. There may be other compounds present within the AGE extract that are responsible for inhibiting agonist-induced platelet aggregation. One possible explanation for the results generated in this study is that the concentration range used is not a true representation of the concentrations actually found within AGE. Another possible reason is that it has not been taken into account the uptake of AGE components via the gastrointestinal tract as this process may have an effect upon the results. Also there is the variation between individuals as it has been found throughout this study that certain volunteers are more susceptible to the actions of both the individual components and their mixtures than others.

Agarwal *et al.* (1977) conducted a clinical trial involving the ingestion of cycloalliin (0.25 g/day). In their trial they found that cycloalliin had a profound effect upon platelet aggregation induced by ADP. In this study we found that cycloalliin inhibited platelet aggregation at all the concentrations used. However, these results were not deemed significant. Thus, this work does not support the findings of Agarwal *et al.* (1977).

It was found that the cysteine sulphoxide mixture was very effective in inhibiting platelet aggregation at relatively low concentrations. It was also observed that the extent of aggregation had a tendency to return to the control level at high concentrations. This was as expected as it has been reported in previous studies (Eyre *et al.* 1983; Macdonald and Langer 2004; Macdonald *et al.* 2000; Morimitsu *et al.* 1992; Yanagita *et al.* 2003) using different garlic preparations that these individual compounds are thought of as some of the main bioactive constituents of garlic. It has also been reported in the literature that these compounds are responsible for garlic's characteristic odour (Morimitsu *et al.* 1992; Wang *et al.* 1999; Xiao and Parkin 2002). However, the results obtained in this study show that the inhibitory properties of AGE are not due to any one of the organosulphur compounds tested. In order for AGE to inhibit agonist-induced platelet aggregation effectively all the constituents must be present.

The results obtained for the three individual amino acids were unexpected, as it was thought that the amino acids L-arginine and L-cysteine would have had a significant effect upon platelet aggregation. Anfossi *et al.* (2001) conducted a study examining the anti-aggregatory effects of L-cysteine in the form of N-acetyl-L-cysteine (NAC). They found that NAC a thiol compound exerts direct anti-aggregatory effects through an increased bioavailability of platelet nitric oxide (NO). It is known that both NO produced by the endothelial cells (Morihara *et al.* 2002) and also by the platelet acts as an inhibitor of platelet aggregation *in vitro*. NO synthesis within platelets is controlled by the amino acid L-arginine as it provides a guanidino nitrogen group for NO synthesis through NO synthase activity (Anfossi *et al.* 2001; Sago *et al.* 2000). This leads to the question does an increase in the availability of these amino acids modulate the response of human platelets in vitro? As AGE is known to contain these particular amino acids and is it possible that the inhibitory effect of AGE is due to the actions of these particular amino acids?

Neither L-arginine nor L-cysteine had a profound inhibitory effect upon platelet aggregation at any of the concentrations used apart from 9.0 mmol/L. The same cannot be said for L-methionine as it was found to be an effective inhibitor of platelet aggregation. However, it may be argued that L-cysteine is not a stable compound and studies that have looked at the effects of this particular amino acid have chosen to use it in the form of NAC, a much more stable compound. Also the amino acids may not have been transported readily across the platelet membrane and that the levels of endogenous L-arginine are more important than the exogenous ones. Even though both of these amino acids are used as supplements the concentrations that may be given orally are significantly higher than those used in this study. Another factor is that prolonged conditioning may result in a reduction of inhibition as 10 min incubation may not have been long enough for noticeable effects to be observed.

Aspirin (acetylsalicyclic acid) is a known inhibitor of platelet aggregation and has been used clinically for a number of years both *in vivo, in vitro* and in the treatment of cardiovascular disease (Awtry and Loscalzo 2000; Chakraborty *et al.* 2003; Clutton *et al.* 2001; Nailin *et al.* 2003; Kong 2004). Aspirin prevents aggregation by irreversibly impeding cyclooxygenase (COX) and reducing thromboxane A_2 (TXA_2) and prostaglandin synthesis (Awtry and Loscalzo 2000; Clutton *et al.* 2001; Ferri *et al.* 1994; Pollack 2003). The majority of *in vitro* studies have used agonists such as collagen and thrombin to stimulate platelet aggregation. There have not been many studies that have utilised ADP. In one such study Chakraborty *et al.* (2003) found that aspirin can inhibit ADP-induced platelet aggregation. As this study is involved with the mechanisms of inhibition by AGE the most logical approach would be to compare AGE and its inhibitory action to those of known inhibitors. The findings in this study oppose those by Chakraborty in that aspirin did not inhibit ADP-induced aggregation at the concentrations used in their study. It was found in this study that slight inhibition was noted at two of the concentrations used (0.00078 and 0.1 mmol/L respectively) but it was not significant. These results strengthen the argument that the biochemical pathways that result in platelet aggregation via stimulation with ADP are different to those initiated by other agonists. Thus, it can be assumed from this study that the enzyme COX does not play an important role in ADP-induced aggregation and that platelets can be activated by pathways that are not blocked by aspirin (Kong 2004).

Thus, a TXA_2 independent pathway must contribute to platelet aggregation induced by ADP.

However, there may be another explanation for the observed results as it has been found that between 8-45 % of the population are resistant to the effects of aspirin. As the optimal dose required to induce complete inhibition of platelet aggregation is subject to inter-individual variability – the mechanism(s) by which some people are resistant to aspirin *in vivo* remains to be determined (Kong 2004; Macchi *et al.*, 2002).

AGE aggregation was compared to other known classical inhibitors of platelet aggregation (AlF_3, AG527, BIS, GEN, IBMX, NaF, OKA, indomethacin and PGE_1), the inhibitory mechanisms of these classical inhibitors are well established (Table 3.3). The only inhibitors that had any significant inhibitory effect upon ADP-induced platelet aggregation were: GEN, IBMX, indomethacin and PGE_1. Each inhibitor has its own mechanism for inhibiting platelet aggregation. Using this information these pathways will be explored in more detail in chapter 4, as the next logical step in this study would be to examine the biochemical pathways which are inhibited by these four classical inhibitors (protein tyrosine kinase activity, cAMP and cGMP phosphodiesterases and intracellular cAMP levels) to see if AGE exerts its inhibitory effect at these points during platelet activation and subsequent aggregation.

ADP was chosen as the primary agonist for inducing platelet aggregation because the results obtained with the particular agonist were consistent and easily reproducible throughout the study.

The major findings generated in this study are summarised in Figure 3.21.

3.4. SUMMARY OF RESULTS

- AGE inhibits agonist-induced platelet aggregation in freshly prepared human PRP.
- AGE is more effective in inhibiting platelet aggregation induced by the agonists: ADP, adrenaline and thrombin at low percentage concentrations than in aggregation initiated by A23187, AA, collagen and PMA.
- Ethanol does not inhibit ADP-induced platelet aggregation therefore the enzyme PLD has no significant role in platelet aggregation initiated with ADP.
- Ethanol inhibits collagen-induced platelet aggregation in a dose-dependent manner. Therefore the enzyme PLD must play an important role in platelet activation and subsequent aggregation initiated with collagen.
- Isolated components from AGE do not inhibit ADP-induced platelet aggregation.
- A mixture of the nine individual components at their optimum concentrations did not inhibit platelet aggregation significantly compared to the control.
- The amino acids are not very effective inhibitors of ADP-induced platelet aggregation both individually and as a mixture. It was found that L-methionine produced the greatest degree of inhibition.
- A mixture of nine components found in AGE and the three amino acids did not significantly inhibit ADP-induced platelet aggregation.
- Aspirin did not inhibit ADP-induced platelet aggregation, when tested in a concentration-dependent manner. Inhibition was observed at certain concentrations, but tended to follow a biphasic pattern. Therefore, platelet aggregation must occur through pathways that are thromboxane-independent when initiated with ADP.
- The only inhibitors that inhibited ADP-induced platelet aggregation were IBMX, indomethacin, GEN and PGE_1.

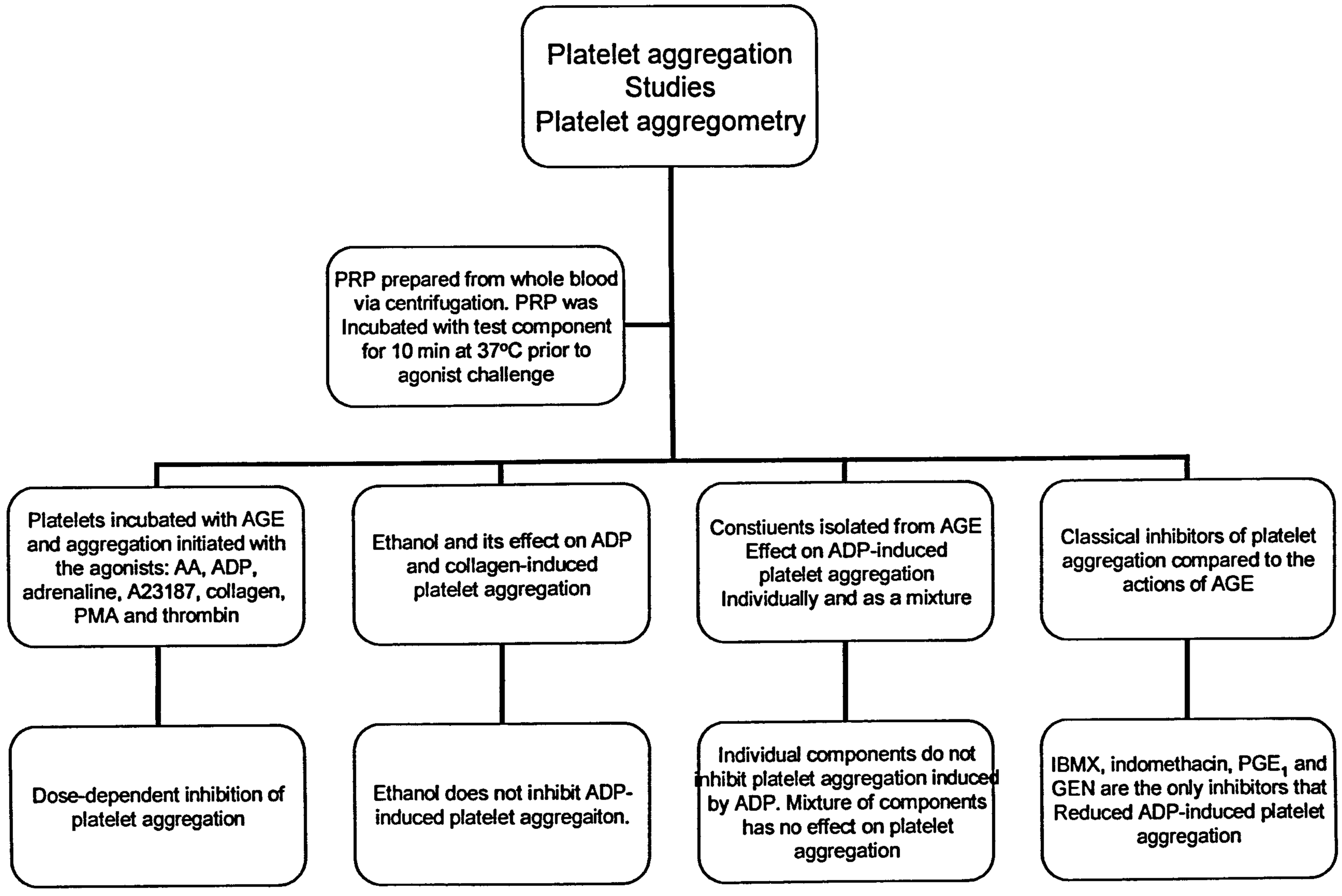


Figure 3.21. Flow chart depicting a summary of the major findings generated from the platelet aggregation studies *in vitro.*

CHAPTER 4
THE INHIBITORY MECHANISMS OF AGE AND ITS EFFECT ON THE BIOCHEMICAL EVENTS THAT INITIATE PLATELET AGGREGATION *IN VITRO*

4. THE EFFECT OF AGE ON SIGNALLING EVENTS DURING PLATELET AGGREGATION

4.1. INTRODUCTION

Platelet activation and subsequent aggregation is an agonist-dependent process i.e. the biochemical signalling pathways involved are dependent upon the stimulating agonist and the class of receptor to which it binds. As mentioned previously (refer to section 1.2.1, Chapter 1) platelet activation and aggregation is a complex biochemical process that requires the cooperation of many signalling pathways in order to bring about the formation of a stable platelet aggregate both *in vitro* and *in vivo*. The main objective of the studies reported in Chapter 3 was to identify possible inhibitory mechanisms for AGE on agonist-induced platelet aggregation by comparing the actions of known platelet aggregation inhibitors to that of AGE using platelet aggregometry.

To reiterate AGE inhibited platelet aggregation induced by a variety of agonists. The only classical inhibitors that significantly inhibited platelet aggregation induced by ADP were, IBMX, GEN and PGE_1. IBMX exerts its inhibitory response through the degradation of both cAMP and cGMP phosphodiesterases leading to an increase in cAMP levels within the platelet. GEN down regulates PTK activity which reduces platelet aggregation, whereas, the prostaglandin PGE_1 exerts its inhibitory effect by increasing the intracellular levels of cAMP within the platelet.

Therefore, it was decided from these results to examine the effects of AGE on the following four pathways involved in platelet aggregation:

- Calcium mobilisation – common to all forms of agonist stimulation. Calcium mobilisation is essential for cytoskeletal rearrangement in platelets from a resting discoid to the formation of pseudopia to allow for aggregate formation.
- AA metabolism – due to the strong indication in the literature of its importance during platelet aggregation. Also clinically available anti-platelet therapies are targeted against COX, an important mediator in AA metabolism.
- Intracellular cAMP levels – levels of cAMP regulate platelet aggregation in that increased levels prevent further aggregation from occurring.
- Fibrinogen binding – the formation of a stable platelet aggregate

The primary reason for choosing the above events that occur during platelet activation/ aggregation is that all of them are associated with events that occur downstream of soluble agonist /receptor interaction (Figure 1.3, page 12). The other reason is that from results obtained in chapter 3 (platelet aggregation studies) AGE; when compared to the classical inhibitors had a similar inhibitory profile to that of PGE_1, GEN, IBMX and indomethacin, when platelet aggregation was initiated with the agonist ADP (for an explanation of ADP-induced platelet aggregation and the signalling pathways activated by this agonist, refer to Figure 1.3, Chapter 1, page 12).

4.1.1. Calcium signalling and platelet activation/aggregation

Changes to the levels of cystolic calcium within aggregating platelets is a critical step in various aspects of platelet activation such as shape change, aggregation, and secretion (Ardlie 1982; Fisher *et al.* 1985; Heemskerk and Sage 1994; Park *et al.* 2004; Sage *et al.* 2000; Sargeant and Sage 1994; Sage 1997; Sage *et al.* 1997). As platelets are non-excitable cells (cells which cannot propagate action potential) the levels of intracellular calcium are increased by platelet agonists via two sources: (1) influx across the plasma membrane through calcium channels, and (2) the release of calcium from intracellular stores contained within the dense tubular system. Agonists bind to receptors coupled to G-proteins on the platelet membrane which stimulates the enzyme PLC, leading to the formation of intracellular second messengers such as IP_3. These second messengers stimulate the release of calcium from intracellular stores (Figure 4.1b) which then regulates the influx of external calcium through calcium-conducting channels within the plasma membrane. The levels of calcium are sustained via PLA_2 activation and the generation of TXA_2 which further propagates platelet aggregation via the binding of fibrinogen to its receptor GPIIb-IIIa (Huang *et al.* 1991; Heemskerk and Sage. 1994; Nesbitt *et al.* 2003; Park *et al.* 2004; Sage *et al.* 2000; Sargeant and Sage 1994; Sage. 1997; Sage *et al.* 1997).

Calcium plays a very important role in platelet aggregation. One possible mechanism by which AGE may inhibit platelet aggregation is that it interferes with calcium mobilisation by either suppressing the influx of this ion into the platelet or by chelation of calcium within the platelet cytosol or extracellular environment. It has been reported previously by Dillon *et al.* (2003) that AGE may chelate calcium ions present within the platelets. In support of this AGE was found to inhibit platelet aggregation induced by

the calcium ionophore A23187 (Section 3.1, Chapter 3, page 58). Hence it can be suggested that the anti-aggregation effect maybe related to an intraplatelet suppression of calcium mobilisation, which in turns activates the PLA_2 pathway to propagate aggregation.

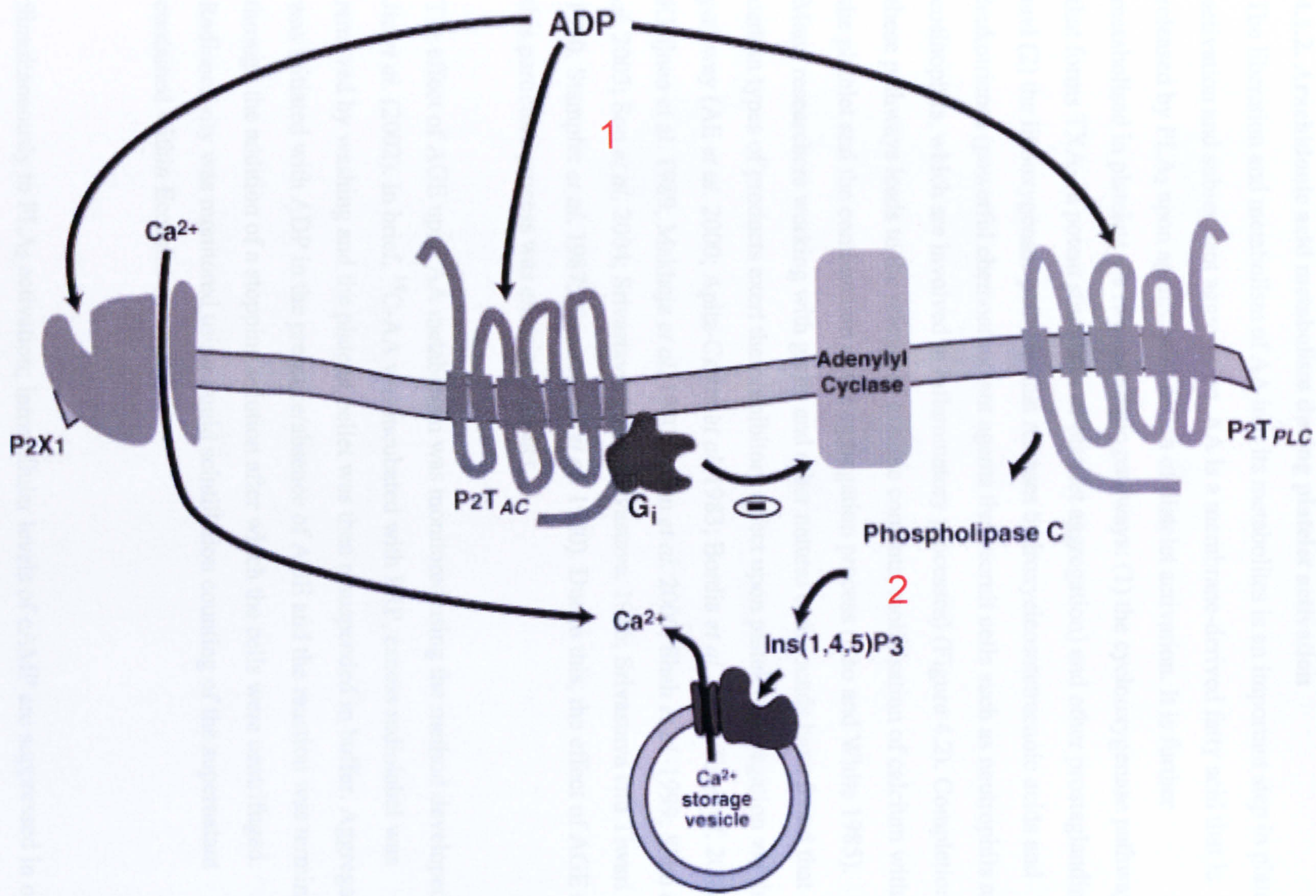


Figure 4.1. Model for the mobilisation of calcium within platelets via the actions of ADP upon its cell surface receptors as proposed by Daniel *et al*. (1999). ADP binds to its receptors and inhibits the actions of adenylyl cyclase (1), at the same time PLC is activated and IP_3 is produced (2) which promotes the release of calcium from intracellular storage vesicles.

4.1.2. Arachidonic acid metabolism during platelet activation

The liberation and metabolism of AA into its metabolites is an important step in platelet activation and subsequent aggregation. AA is a membrane-derived fatty acid that is released by PLA_2 upon agonist stimulation of platelet activation. It is further metabolised in platelets via two metabolic pathways: (1) the cyclooxygenase pathway that forms TXA_2 (a potent stimulator of platelet aggregation) and other prostaglandins, and (2) the lipooxygenase pathway that releases hydroxyeicosatetraenoic acids and leukotrienes (powerful chemoattractant agents that recruit cells such as neutrophils and eosinophils, which are involved in inflammatory processes) (Figure 4.2). Completion of these pathways leads to the release of granule contents, mobilisation of calcium within the platelet and the continuation of the aggregation process (Rao and White 1985). Many researchers working with garlic and other natural compounds have found that certain types of products exert their inhibitory effect upon platelet aggregation via this pathway (Ali *et al.* 2000; Apitz-Castro *et al.* 1983; Bordia *et al.* 1978; Jin *et al.* 2005; Kleijnen et al. 1989; Makheja *et al.* 1980; Moon *et al.* 2000; Shah *et al.* 1999; Sheu *et al.* 2003; Son *et al.* 2004; Srivastava. 1984; Srivastava. 1986; Srivastava and Tiwari 1980; Stampfer *et al.* 1987; Vanderhoek *et al.* 1980). Due to this, the effect of AGE on this particular process was examined further.

The effect of AGE upon AA metabolism was monitored using the method developed by Jin *et al.* (2002). In brief, ^{14}C-AA was incubated with PRP, excess radiolabel was removed by washing and the platelet pellet was then resuspended in buffer. Aggregation was initiated with ADP in the presence/absence of AGE and the reaction was terminated through the addition of a stopping solution after which the cells were centrifuged. Radioactivity was monitored using liquid scintillation counting of the supernatant contained within Ecoscint A.

Simultaneously to PLA_2 activation; intracellular levels of cAMP are suppressed in order for platelet aggregation to continue unabated.

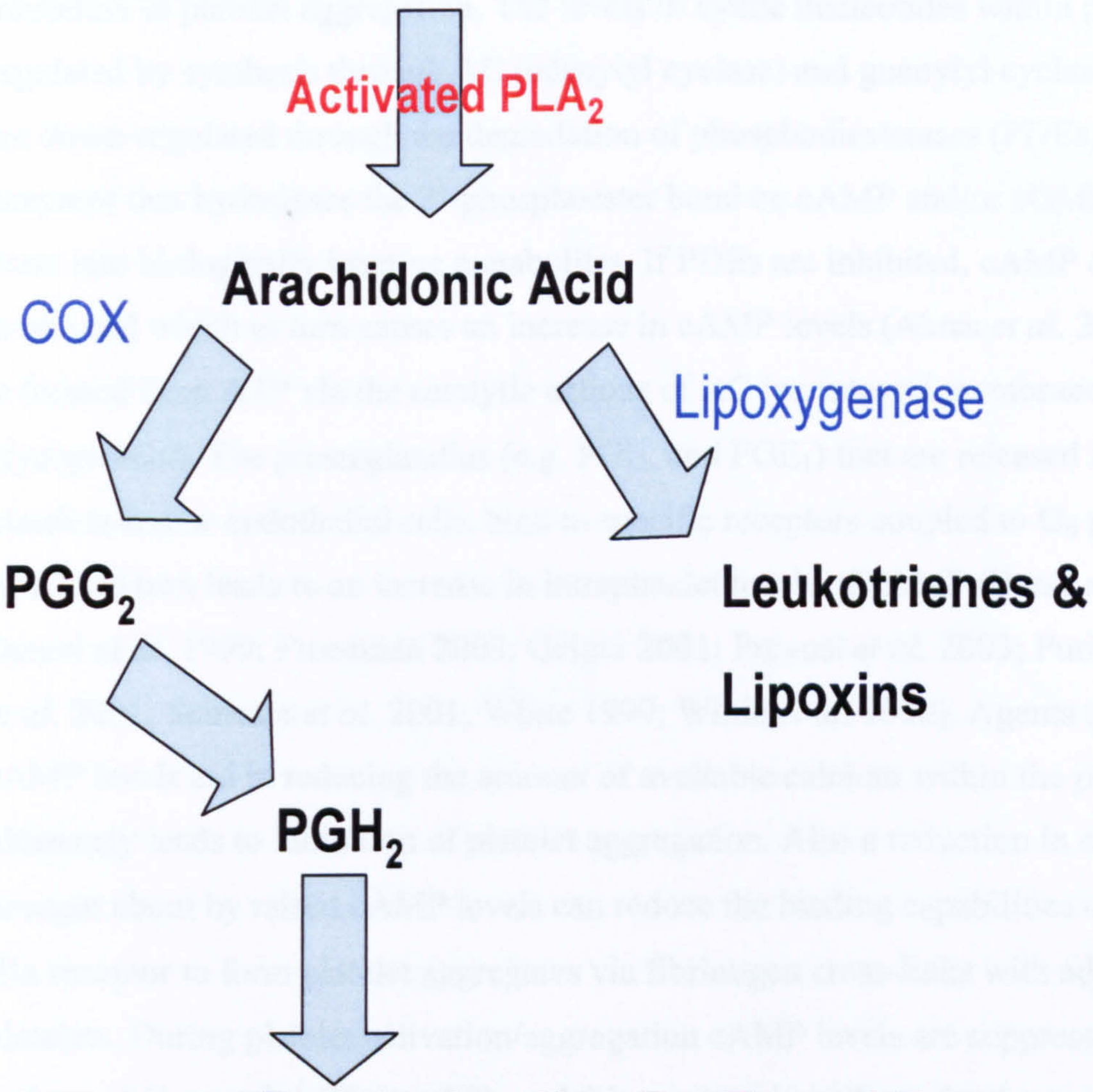


Figure 4.2. A schematic representation of the AA metabolic pathway. Agonist receptor interaction stimulates the release of AA by PLA_2. AA is metabolised further by two separate pathways (1) the enzyme COX into the prostaglandins D_2, E_2 and $F_{2\alpha}$ and, (2) the lipooxygenase pathway which releases hydroxyeicosatetraenoic acids and leukotrienes which promotes further platelet aggregation and activation.

4.1.3. cAMP and platelet aggregation

The cyclic nucleotides regulate platelet function by controlling the amount of platelet aggregation that occurs *in vivo*; and include cAMP and cGMP which are both potent inhibitors of platelet aggregation. The levels of cyclic nucleotides within platelets are regulated by synthesis through AC (adenylyl cyclase) and guanylyl cyclase (GC) and are down-regulated through the degradation of phosphodiesterases (PDEs) – a family of enzymes that hydrolyses the 3'-phosphoester bond on cAMP and/or cGMP, converting them into biologically inactive metabolites. If PDEs are inhibited, cAMP decomposition is reduced which in turn causes an increase in cAMP levels (Aktas *et al.* 2002). cAMP is formed from ATP via the catalytic actions of AC (an integral membrane glycoprotein). The prostaglandins (e.g. PGI_2, and PGE_1) that are released from either platelets and/or endothelial cells, bind to specific receptors coupled to G_S proteins which, in turn leads to an increase in intraplatelet levels of cAMP (Brass *et al.* 2004; Daniel *et al.* 1999; Freedman 2003; Geiger 2001; Prevost *et al.* 2003; Puri 1999; Savage *et al.* 2001; Schwarz *et al.* 2001; White 1999; Wilde *et al.* 2000). Agents that raise cAMP levels aid in reducing the amount of available calcium within the platelet which ultimately leads to inhibition of platelet aggregation. Also a reduction in calcium brought about by raised cAMP levels can reduce the binding capabilities of the GPIIb-IIIa receptor to form platelet aggregates via fibrinogen cross-links with adjacent platelets. During platelet activation/aggregation cAMP levels are suppressed via the actions of G_I proteins (Figure 4.3), and this suppression allows for the aggregation process to continue unabated.

Platelet aggregation (fibrinogen-receptor interaction to form a stable platelet aggregate) is dependent upon the levels of cAMP within the platelet.

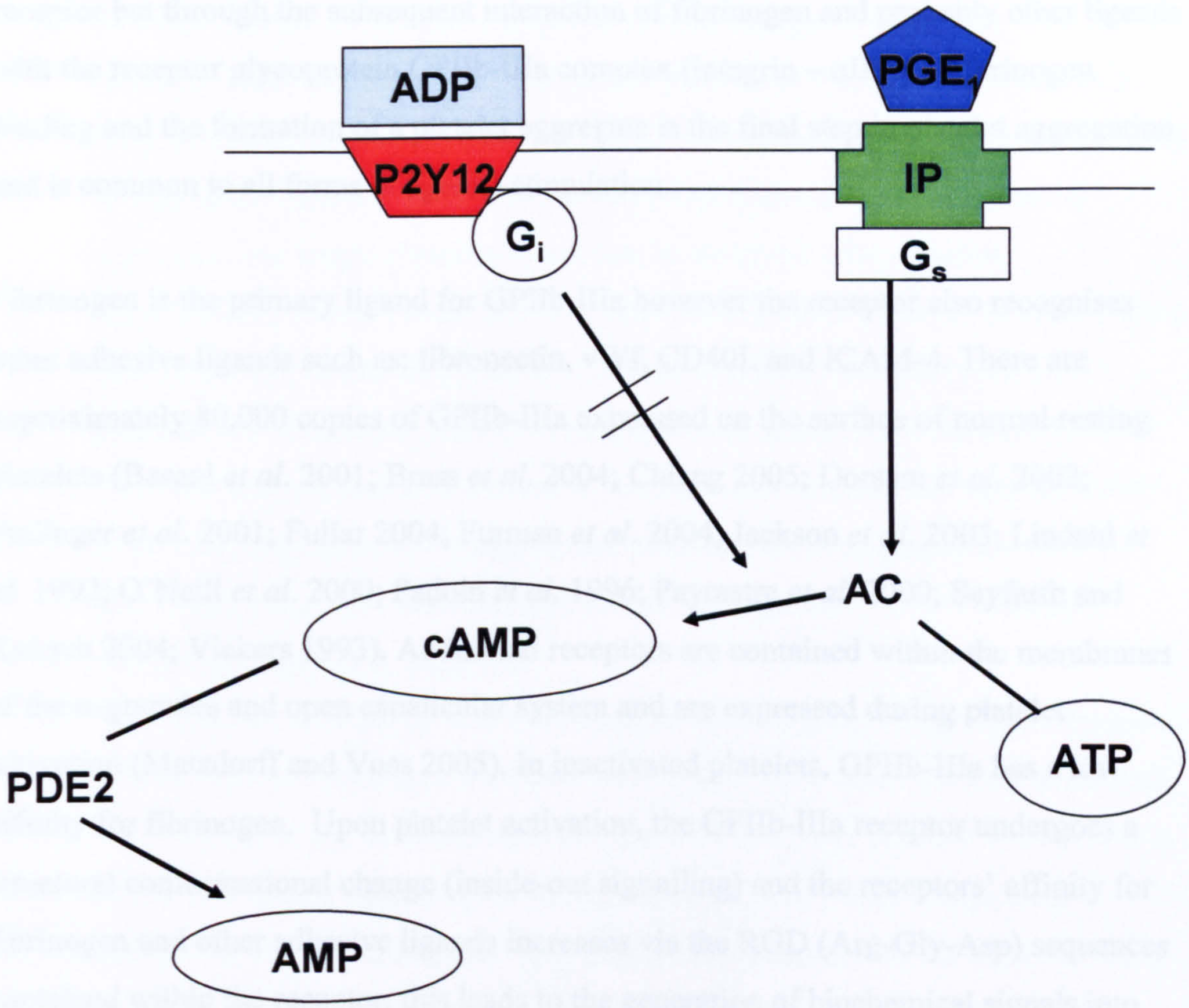


Figure 4.3. The events surrounding cAMP formation and suppression during platelet aggregation. ADP mediates platelet aggregation by receptors coupled to G-protein mediated pathways. The receptor P2Y12 inhibits AC activity via G_I allowing for platelet activation and aggregation to occur. PGE_1 binds to IP receptor which is coupled to a G_S protein. This stimulates AC activity to increase levels of cAMP, thus inhibiting platelet aggregation. cAMP is degraded into AMP via PDE2.

4.1.4. Fibrinogen binding and platelet aggregation

In agonist induced platelet aggregation, the action of the agonist on the generation of the signal that stimulates granule secretion is not caused by the binding of the agonist to its receptor but through the subsequent interaction of fibrinogen and probably other ligands with the receptor glycoprotein GPIIb-IIIa complex (integrin – $\alpha IIb\beta_3$). Fibrinogen binding and the formation of a platelet aggregate is the final step in platelet aggregation and is common to all forms of agonist stimulation.

Fibrinogen is the primary ligand for GPIIb-IIIa however the receptor also recognises other adhesive ligands such as: fibronectin, vWf, CD40L and ICAM-4. There are approximately 80,000 copies of GPIIb-IIIa expressed on the surface of normal resting platelets (Basani *et al.* 2001; Brass *et al.* 2004; Chiang 2005; Dorsam *et al.* 2002; Frelinger *et al.* 2001; Fullar 2004; Furman *et al.* 2004; Jackson *et al.* 2003; Lindahl *et al.* 1992; O'Neill *et al.* 2000; Padoin *et al.* 1996; Payrastre *et al.* 2000; Seyfarth and Koksch 2004; Vickers 1993). Additional receptors are contained within the membranes of the α-granules and open canalicular system and are expressed during platelet activation (Matzdorff and Voss 2005). In inactivated platelets, GPIIb-IIIa has a low affinity for fibrinogen. Upon platelet activation, the GPIIb-IIIa receptor undergoes a structural conformational change (inside-out signalling) and the receptors' affinity for fibrinogen and other adhesive ligands increases via the RGD (Arg-Gly-Asp) sequences contained within the receptor, this leads to the generation of biochemical signals into the platelet (outside-in signalling), that further drives the aggregation process. These include an increase in tyrosine phosphorylation of signalling proteins such as Syk (a cytosolic nonreceptor tyrosine kinase involved in signal transduction processes such as an increase in Ca^{2+} mobilisation) and FAK (Focal Adhesion Kinase; where actions are mediated through outside-in signalling via the conformational change in the GPIIb-IIIa receptor, its function is in cytoskeletal reorganisation) (Buensuceso *et al.* 2005; de Virgilio *et al.* 2004; Hers *et al.* 1997); increase in calcium mobilisation (Nesbitt 2003; Quinton *et al.* 2002; Rosado *et al.* 2001), calpain activation and increased cytoskeletal organisation (platelets change from resting discoid to the formation of pseudopodia on the platelet surface) (Eckly *et al.* 2002; Levy-Toledano *et al.* 1997) GPIIb-IIIa receptors on adjacent platelets link together via fibrinogen cross-bridges to form platelet aggregates (Figure 4.4).

To monitor the effects of AGE upon fibrinogen binding during ADP-induced platelet aggregation, four independent methods were used as outlined below:-:

1. The first method investigated involved platelet disaggregation. The platelet inhibitor PGE_1 had comparable inhibition profiles to that of AGE (Chapter 3). Another physiological characteristic of this prostaglandin is that when it is added to aggregated platelets it can reverse this process and disperse the platelet aggregate into single platelets which can be observed using platelet aggregometry (Kikura *et al.* 2002). Thus, it was decided to see if AGE acted in a similar manner to that of PGE_1 with regards to platelet disaggregation. To monitor this occurrence platelet aggregation was initiated via the addition of ADP and the reaction was allowed to run for 5 min in a platelet aggregometer. AGE was then added to the aggregated platelets and disaggregation was monitored using light transmittance platelet aggregometry (For a detailed method see Chapter 2, page 40).
2. A simple adhesion assay was developed to monitor platelet aggregate formation to immobilised fibrinogen. Platelets were loaded with either the biological stain Rose Bengal or the radiolabelled chromium-51 and were allowed to adhere to a fibrinogen-coated surface upon stimulation with ADP. Two assays were conducted: direct and indirect. The indirect assay monitored the effect of AGE treated platelets and their ability to bind to fibrinogen following agonist challenge. The direct assay examined the effect of AGE directly upon the immobilised fibrinogen molecule (For a detailed method refer to chapter 2, page 42).
3. The simple adhesion assay produced results which suggested that pre-treatment of platelets with AGE reduced platelet aggregate formation. However, the method was very basic one and the results are not very consistent. Hence, a well established method was utilised: Flow cytometric analysis. This particular method analyses the binding capabilities of the fibrinogen receptor GPIIb/IIIa to its ligand fibrinogen through the use of antibodies specific for both platelets and the receptor GPIIb/IIIa (See page 42 for a detailed method outline).
4. SEM imaging was used to monitor platelet aggregation in the presence/absence of AGE (refer to page 47, chapter 2 for a detailed method of the procedure).

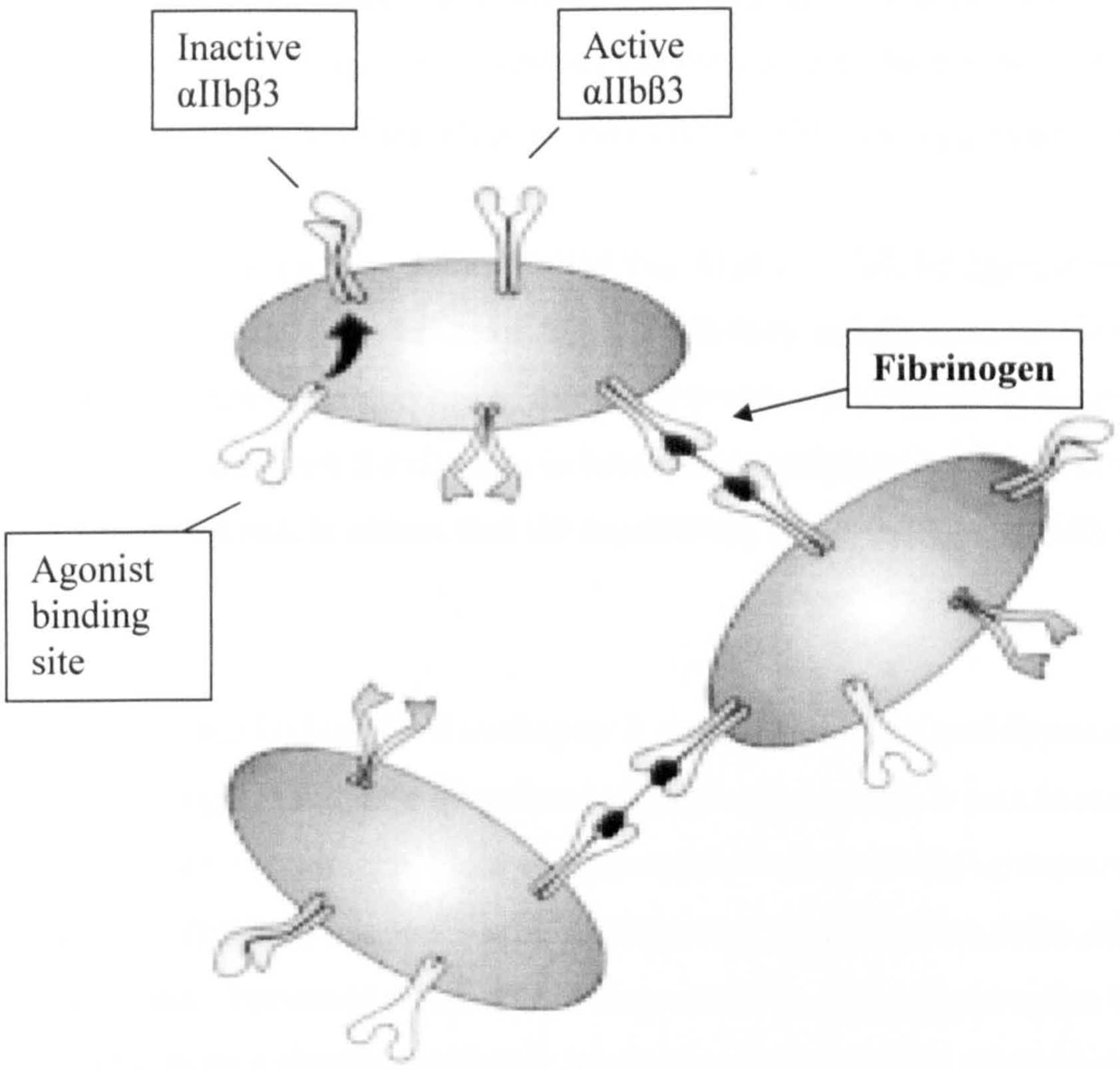


Figure 4.4. Traditional model of platelet aggregation. Soluble agonists bind to one or more receptor on the platelet cell surface, initiating signalling events that are linked with the integrin αIIbβ3 receptor activation. The active form of the receptor binds to fibrinogen, linking adjacent cells to form a stable aggregate (Dopheide et al. 2001).

4.2. RESULTS

4.2.1. The effect of AGE on calcium mobilisation during agonist-induced platelet aggregation

I. Measurement of intracellular calcium in activated and inactivated platelets

Platelet aggregation is triggered by a variety of extracellular signals and many agonists interact with the receptors coupled to PLC, leading to the formation of DAG and IP_3 which in turn releases calcium from intracellular stores. The emptying of these calcium stores regulates calcium-conducting channels within the plasma membrane of the platelets, thus initiating the influx of external calcium ions into the platelets. This sustained level of calcium activates PLA_2 resulting in the synthesis of TXA_2 and the subsequent release of secretory granules to complete the aggregation process.

Although it is has been demonstrated that AGE may inhibit agonist-induced platelet aggregation, the direct effect of AGE on calcium mobilisation within the platelets is unknown. The main purpose of this experiment was to design a procedure that will effectively measure the changes in intracellular calcium in both inactivated (as a negative control, to ensure that the experiment was working correctly) and activated platelets.

Using the method outlined in chapter 2, page 40, calcium mobilisation was monitored in both aggregated and non-stimulated platelet suspensions. It was found during these preliminary experiments (data not shown) that a Fura-2/AM concentration of 1 μM was not sufficient to produce a strong fluorescence signal that could be measured by the fluorimeter. However a Fura-2/AM concentration of 2 μM proved to be more effective in generating a significant signal. It was also noted that the original concentration of the ionophore A23187 (2 μM) needed to induce activation was not effective and that a concentration of 5 μmol/L was required. Platelets were then loaded with Fura-2/AM at a final concentration of 2 μM, both $CaCl_2$ (an external source of Ca^{2+}) and EGTA (a Ca^{2+} chelator) were then added individually and together to the platelet suspensions prior to agonist-induced activation at a final concentration of 1 mM. The calcium ionophore A23187 was used to initiate platelet aggregation and calcium influx at a concentration of 5 μM. In these experiments it was noted that external calcium is important and essential for platelet aggregation. As platelets that had not been exposed to an external

calcium ion source displayed a reduction in fluorescence intensity and ultimately a reduction in the calcium concentration within the platelet.

From the experiments reported above it lead to the question can AGE inhibit agonist-induced platelet aggregation by reducing the amount of calcium available to the aggregating platelets through chelation of calcium ions? The following experiment was performed to answer this question.

Fura-2/AM loaded platelets at a concentration of 2 μM were incubated with $CaCl_2$ at a final concentration of 1 mM and 25 % (v/v) AGE for 10 min at room temperature. A23187 at a final concentration of 5 μM was then added to the AGE treated platelets and the fluorescence was measured. It was found that AGE significantly decreased the calcium concentration when compared to the control (inactivated platelets). The experiment was repeated again with $CaCl_2$ being substituted for EGTA (a chelator of metal ions was used to determine if external calcium ion influx is important in maintaining agonist-induced platelet aggregation) at a final concentration of 1 mM. However, the results obtained were not viable due to the large negative values that were recorded by the fluorimeter. The addition of $CaCl_2$ to platelets acts as an external source of calcium ions. The purpose of this experiment was to observe if AGE had the potential to reduce the availability of external calcium ions to the aggregating platelets, as both internal and external sources of calcium ions are important for maintaining platelet aggregation. These results indicate that platelet aggregation was reduced when AGE was added to platelets that had been pretreated with $CaCl_2$.

It is known from the experiments conducted by Dillion *et al.*(2003) that AGE has metal chelating properties and it is likely that AGE interferes with calcium mobilisation and subsequent inhibition of platelet aggregation. To ascertain whether AGE is really having an effect upon calcium influx and mobilisation within the platelet; the following points were examined:

- EDTA used instead of EGTA as it is more specific for calcium – the above experiment suggested that external calcium ion influx is an important factor in platelet aggregation. In that, the addition of EGTA to platelet suspensions resulted in a decrease in platelet aggregation. The actions of EDTA will be

compared to that AGE to see if AGE has potential calcium ion chelating properties.

- The ability of Fura-2/AM loaded platelets to aggregate in the presence/absence of external calcium was investigated. Aggregation was monitored using platelet aggregometry and compared to the control for aggregation induced by ADP. Platelet aggregometry is a good indicator of platelet aggregation therefore, it is essential to determine that AGE is directly affecting calcium mobilisation in the presence/absence of calcium ions.

The results generated in this study show that platelets from different individuals, when stimulated with the agonists ADP and A23187, display variable aggregation and calcium mobilisation when compared to the PBS controls therefore, typical individual data is presented in this study. Figure 4.5 shows that ADP when added to platelet suspensions caused approximately 70% aggregation compared to the control sample. In the presence of Fura-2/AM, platelet aggregation induced by ADP was lower than platelets that had not been exposed to the Fura-2/AM (Figure 4.5). The addition of calcium ions to the platelets resulted in a decrease in the aggregation response, compared to the Fura-2/AM loaded platelets in the absence of calcium ions. The addition of AGE and calcium to the platelets resulted in a reduction in aggregation that was similar to Fura-2/AM loaded platelets in the presence of both external calcium ions and EGTA (Figure 4.5).

Experiments that monitored calcium mobilisation showed that addition of ADP resulted in an increase in calcium ions, such that a peak was observed at approximately 0.5 min (Figure 4.6). This result supports earlier studies which showed that an increase in intracellular calcium concentration is indicative of platelet aggregation (Heemskerk and Sage 1994; Sage *et al.* 2000; Sage *et al.* 1997; Sage *et al.* 1997; Sargeant and Sage 1994). The inclusion of AGE resulted in the significant inhibition of platelet aggregation and suppression of calcium ions. The results indicate that the pre-incubation of platelets with AGE had significantly lower calcium ion concentrations implying that AGE may be acting as a possible calcium ion chelator (Figure 4.6).

The calcium ionophore A23187 also induced platelet aggregation with a concomitant increase in the intracellular calcium ion concentration, again the rise in calcium ion

concentration was apparent at 0.5 min (Figure 4.7), this agonist was used as a positive control as it induces calcium ion influx to initiate platelet activation and subsequent aggregation. The effects of AGE upon A23187-induced platelet aggregation were very similar to those observed with ADP. Thus, platelet aggregation was significantly reduced as was the increase in intracellular calcium ion concentration (Allison *et al.* 2006). A23187 is an ionophore that promotes the influx of calcium ions into platelets (Connor *et al.* 2001) and it is possible that AGE interferes with this particular step or steps precedent to the generation of second messengers during platelet activation and aggregation or it simply chelates the available Ca^{2+}.

The activities of EGTA (a known calcium chelator) were compared to that of AGE. It was observed that EGTA at very high concentrations suppressed calcium mobilisation (results not shown). In this experiment there was no evidence of either light or fluorescence quenching by AGE.

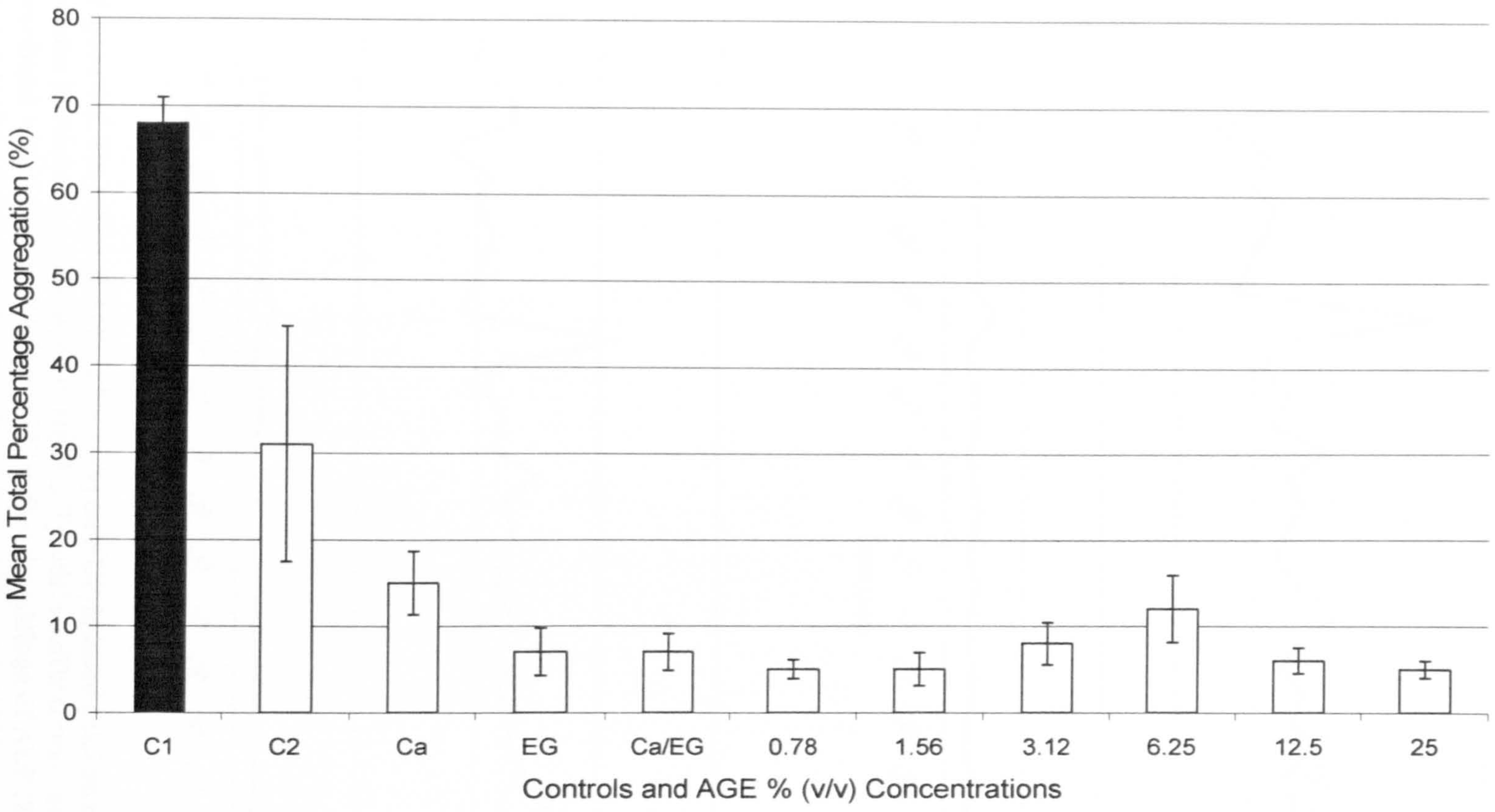


Figure 4.5. The effect of AGE on ADP-induced calcium mobilisation. Fur-2/AM loaded platelets were suspended in HEPES-Tyrode's buffer and incubated with various AGE concentrations (0.78-25 % (v/v)) for 10 min at 37°C; ADP was added to the samples to initiate platelet aggregation which was measured using an aggregometer. Key: (C1) without Fura or AGE; (C2) Fura loaded platelets, no AGE; (Ca) Fura loaded platelets in the presence of 1 mmol/L $CaCl_2$; (EG) Fura loaded platelets in the presence of 1 mmol/L EGTA; (Ca/EG) Fura loaded platelets in the presence of $CaCl_2$ and EGTA; (AGE concentrations) Fura loaded platelets pre-treated with various AGE concentrations in the presence of $CaCl_2$ (Results reproduced from Allison *et al.* 2006).

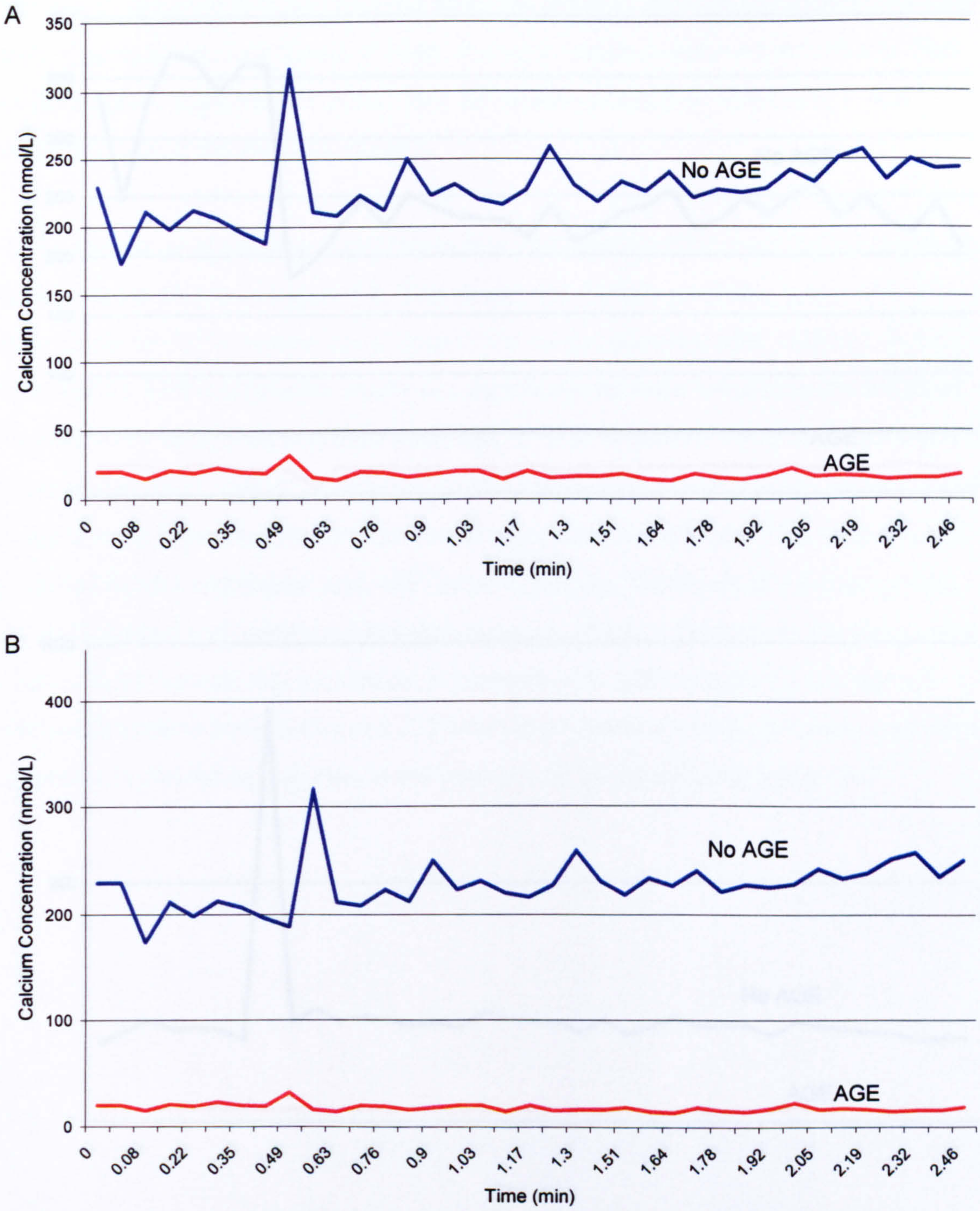


Figure 4.6. The effect of AGE, 25 % (v/v) on ADP-induced calcium mobilisation. Fura-loaded platelets were suspended in HEPES-Tyrode's buffer containing $CaCl_2$ (1 mmol/L) and incubated with AGE for 10 min at 37°C before the addition of ADP (8 µmol/L). Key: (A) donor 1; (B) donor 2 (Results reproduced from Allison *et al*. 2006)

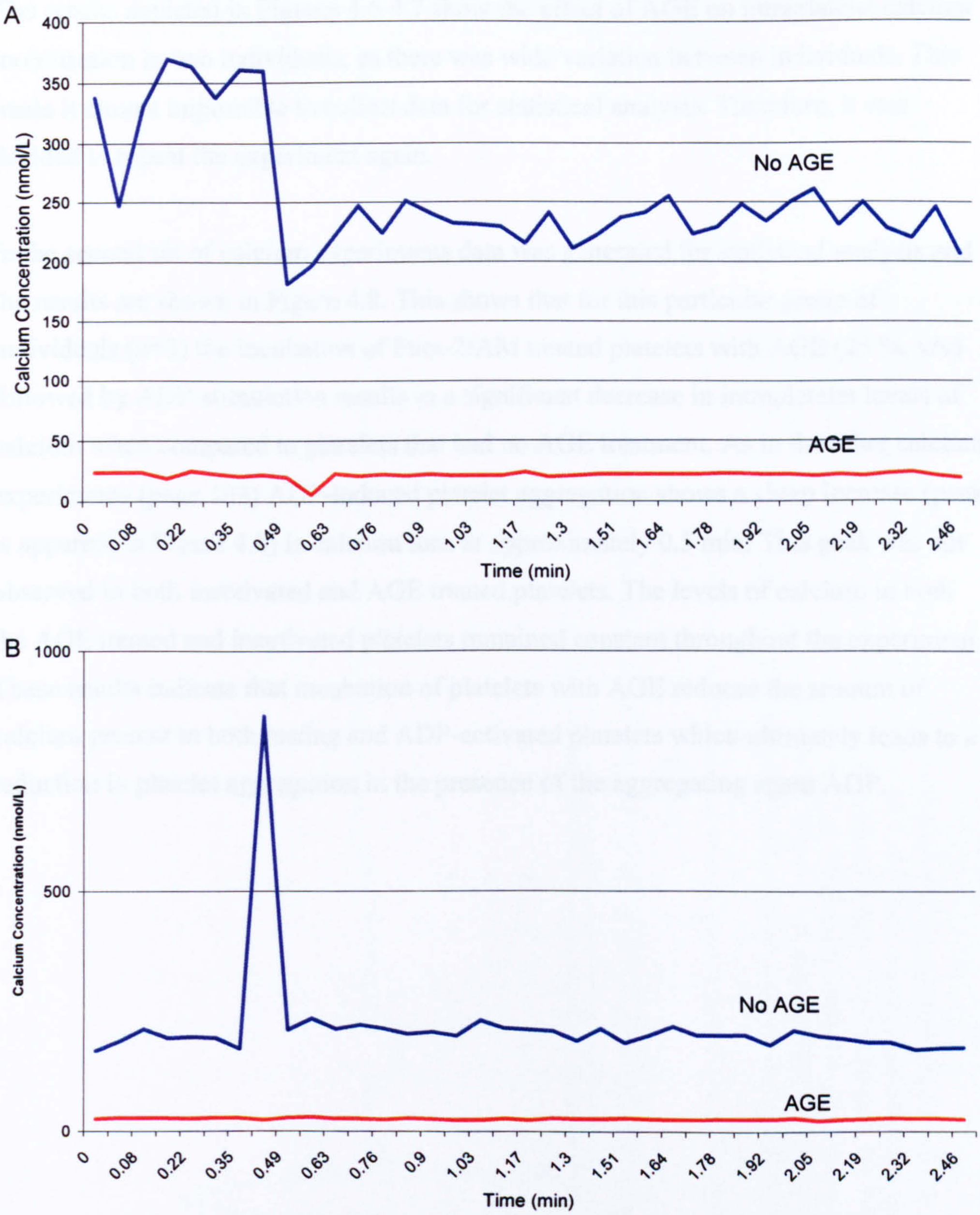


Figure 4.7. The effect of AGE, 25 % (v/v) on A23187-induced calcium mobilisation. Fura-loaded platelets were suspended in HEPES-Tyrode's buffer containing $CaCl_2$ (1 mmol/L) and incubated with AGE for 10 min at 37°C before the addition of A23187 (5 μmol/L). Key: (A) donor 1; (B) donor 2 (Results reproduced from Allison *et al*. 2006).

The results depicted in Figures 4.6-4.7 show the effect of AGE on intraplatelet calcium mobilisation in two individuals, as there was wide variation between individuals. This made it almost impossible to collect data for statistical analysis. Therefore, it was decided to repeat the experiment again.

In the second set of calcium experiments data was generated for statistical analysis and the results are shown in Figure 4.8. This shows that for this particular group of individuals (n=3) the incubation of Fura-2/AM treated platelets with AGE (25 %, v/v) followed by ADP-stimulation results in a significant decrease in intraplatelet levels of calcium when compared to platelets that had no AGE treatment. As in the other calcium experiments (page 103) ADP-induced platelet aggregation shows a sharp increase (peak is apparent in Figure 4.8) in calcium ions at approximately 0.5 min. This peak was not observed in both inactivated and AGE treated platelets. The levels of calcium in both the AGE treated and inactivated platelets remained constant throughout the experiment. These results indicate that incubation of platelets with AGE reduces the amount of calcium present in both resting and ADP-activated platelets which ultimately leads to a reduction in platelet aggregation in the presence of the aggregating agent ADP.

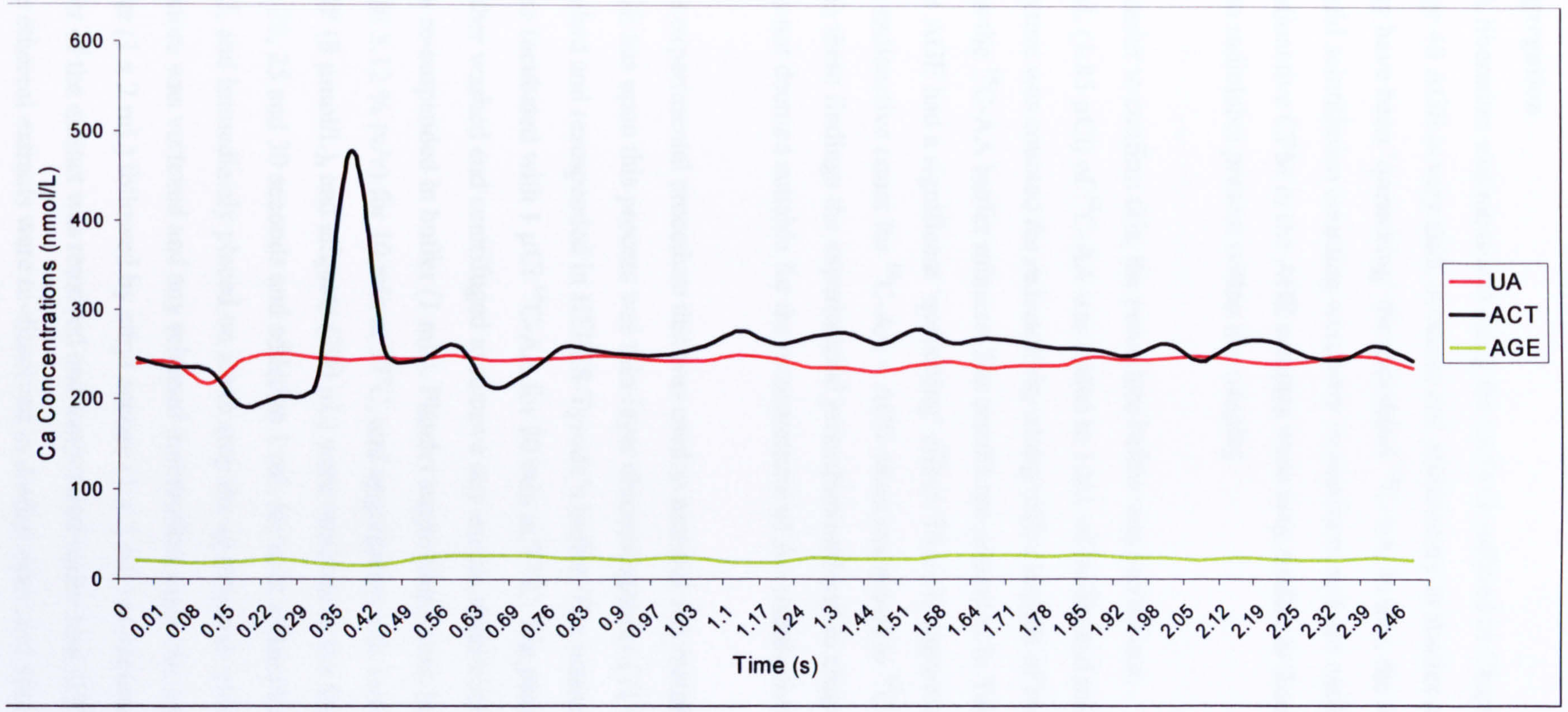


Figure 4.8. The effect of AGE on calcium mobilisation during platelet aggregation initiated with ADP *in vitro.* Platelets were loaded with the calcium indicating dye Fura-2/AM for 45 min at 37°C. The platelets were washed and resuspended in HEPES-Tyrode's buffer. Platelets were stimulated with the agonist ADP and the fluorescence intensity was monitored for 3 min (ACT). In another set of experiments platelets were incubated with AGE, 25% (v/v) for 10 min at 37°C, and aggregation was initiated via the addition of ADP (AGE). Calcium fluorescence was also monitored in unactivated platelets (UA). The results are expressed as means, n=3. There is statistical significance ($P<0.05$) between the AGE treated platelets and platelets activated with ADP alone. KEY: UA – inactivated platelets; ACT – activated platelets; AGE – platelets pretreated with AGE and aggregation initiated with ADP.

4.2.2. The effect of AGE on AA metabolism during ADP-induced platelet aggregation

AA liberation was measured using the method outlined in Chapter 2, section 2.4.2., page 40 AGE is very dark in colour and preliminary in studies a concern was that AGE may have been 'quenching' the radiolabel ^{14}C-AA, in that, the readings obtained via liquid scintillation counting were very inconsistent and not easily reproducible (radioactive CPM in the AGE samples were very similar to those of the negative control – no radiolabel present within the sample).

In order to confirm this, the procedure below was carried out:

1 μL (1.85 μCi) of ^{14}C-AA was added to 1 mL of buffer and mixed. 10 μL of this mixture was counted for radioactivity along with a sample of buffer, a sample of AGE plus the ^{14}C-AA buffer mixture. The results are presented in Table 4.1. and it is clear that AGE had a significant 'quenching' effect. There is approximately 80 % reduction in the radioactive count for ^{14}C-AA + AGE when compared to ^{14}C-AA on its own. Thus, with these findings the experimental procedure outlined in chapter 2 was discarded as it was not deemed suitable for the measurement of AA metabolism.

The experimental procedure that was used to monitor AA metabolism and the effect AGE has upon this process was thin-layer chromatography (TLC). Platelets were washed and resuspended in HEPES-Tyrode's buffer. The washed platelet suspensions were incubated with 1 μCi ^{14}C-AA for 20 min at 37°C. The platelet suspension was then further washed and centrifuged to remove any excess radiolabel and the platelet pellet was re-suspended in buffer (1 mL). Platelet suspensions were incubated with or without AGE 3.12 % (v/v) for 10 min at 37°C, and aggregation was initiated via the addition of ADP (8 μmol/L), and aliquots (200 μL) were removed at the following time points: 0, 10, 20, 25 and 30 seconds and added to 1 mL ice cold saline (4 mM) pH to 3 by use of HCl, and immediately placed on ice to stop the aggregation process: The platelet mixture was vortexed and any released metabolites were extracted using cold diethyl ether (1 x 2 mL) followed by ethyl acetate (1 x 2 mL) extraction. The top colourless layer of the extract was removed and evaporated under heat (55°C) using nitrogen gas. The ethereal extracts were re-dissolved in diethyl ether and spotted onto glass-backed silica Gel G TLC plates along with sodium arachidonate and the prostaglandins D_2, E_2, and $F_{2\alpha}$ as standards. The TLC plates were developed using: hexane/diethyl ether/ acetic

acid (80:20:1, v/v/v). After development the plates were allowed to dry and stained with iodine. However there was insufficient separation following iodine staining of the prostaglandins (no spots were present on the TLC plate to measure Rf values). The entire lanes for each each standard was scraped and measured for radioactivity using liquid scintillation counting.

It was found in this study that platelets pre-labelled with ^{14}C-AA when incubated with AGE and challenged with the agonist ADP showed no significant inhibitory effect upon AA metabolism when compared to the control sample. This was determined using liquid scintillation counting – no significant increases in radiolabelled metabolites were identified. A possible explanation for the observed result is that AA on its own is an agonist and activation may have occurred. Thus, adding ADP would not stimulate aggregation if it has already been initiated.

Table 4.1. The 'quenching' effect of AGE on liquid scintillation counting.

Platelet suspensions labelled with ^{14}C-AA and pretreated with AGE prior to agonist stimulation resulted in 'quenching' during liquid scintillation counting. AGE in the presence of ^{14}C-AA reduced the CPM by 80 % compared to ^{14}C-AA on its own.

Sample	^{14}C - Counts per minute (CPM)
Buffer	22
3.12 % AGE	22
^{14}C-AA	1756
^{14}C-AA + AGE	363

There were numerous problems encountered with this particular method and it resulted in significant variation in the data. Hence this method was also discarded. The major problem in this experiment was that significant amounts of the radiolabel was lost during the washing step(s) and solvent extraction process.

The above method was modified in that platelet-rich plasma was pre-incubated in the presence/absence of AGE for 10 min at 37°C. Aggregation was stimulated by the addition of ADP and the aggregatory process was allowed to run for approximately 30 seconds. The reaction was terminated by placing the PRP on ice followed by centrifugation at 1500 g at 4°C for 10 min. The supernatant was then removed and the platelet pellet resuspended in HEPES-Tyrode's buffer. Aliquots (1 mL) of the platelet suspension were homogenised on ice using a Donce Homogeniser. The platelet homogenate was equilibrated to 37°C for 5 min, after which, 2 mM $CaCl_2$ was added to the platelet lysate, immediately followed by the addition of ^{14}C-AA and unlabelled AA (1 μCi and 1 μM, respectively). Aliquots (100 μL) were removed at the following time intervals: 0, 0.5, 1, 2, 3, 5, 10 and 15 min and placed into 1 mL ice-cold buffer and 1 mL ethyl acetate. The mixture was vortexed and centrifuged at 2000 g for 10 min at 4°C. The organic layer was removed and dried at a temperature of 55°C under nitrogen gas. Finally the residues were re-dissolved in ethanol and spotted onto glass-backed silica gel G TLC plates. Prostaglandins D_2, E_2, $F_{2\alpha}$ and sodium arachidonate standards were spotted separately (Table 4.2). The plates were developed in: hexane/diethyl ether/ acetic acid (80:20:1, v/v/v) to a distance of 15 cm and plates were stained with iodine, the spots identified, scraped and measured for radioactivity.

The following changes were made to the experiment: the development system used above provided data, however, it was found from an intensive literature search that the following system is more widely used and was used in futher experiments.

chloroform/methanol/acetic acid/water (100/15/125/1, v/v/v/v) – (Bailey *et al.* 1977)

Table 4.2 displays the Rf values obtained for the standards using the developing system: chloroform/methanol/acetic acid/water (100/15/125/1, v/v/v/v). The Rf value for standards are similar to those published by Bailey *et al.* (1977).

The results obtained in this study (Figure 4.9) show that arachidonic acid metabolism does not, play a major role in platelet aggregation induced by the agonist ADP *in vitro*. AGE did not inhibit the incorporation of ^{14}C-AA into platelets, uptake of approximately 85 % occurred. The levels of labelled AA present in AGE treated samples were very similar to that of the controls indicating, that no AA metabolism had taken place upon ADP-induced platelet aggregation. A possible reason for the observed result is that the addition of unlabelled AA plus labelled AA may have resulted in the unlabelled AA being taken up into the platelet rather than the labelled AA. A similar result was obtained for the agonist thrombin. Thus, the type of cell-signalling pathway that initiates platelet aggregation is dependent upon the stimulatory agonist used to induce platelet aggregation. Hence, ADP-induced platelet aggregation does not rely solely upon AA metabolism for platelet aggregation to occur.

This experiment was modified a number of times as shown by the 3 methods used in this study, and was conducted numerous times in the presence of another agonist thrombin, and each time it was found that AA metabolism does not play a significant role in ADP-induced platelet activation and aggregation.

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This experiment was modified a number of times as shown by the 3 methods used in this study, and was conducted numerous times in the presence of another agonist thrombin, and each time it was found that AA metabolism does not play a significant role in ADP-induced platelet activation and aggregation.

Table 4.2. Rf values for the standards Prostaglandins D_2, E_2, $F_{2\alpha}$ and unlabelled sodium arachidonate using the following development system chloroform: methanol: acetic acid: water (100/15/12.5/ 1, v/v/v/v). The Rf values for the standards were calculated by measuring the distance of the compound moved to the centre of the smear/spot and dividing the value by the distance moved by the solvent front (15 cm).

Standards	Rf Values
Prostaglandin D_2	0.18
Prostaglandin E_2	0.16
Prostaglandin $F_{2\alpha}$	0.1
Sodium arachidonate	0.61

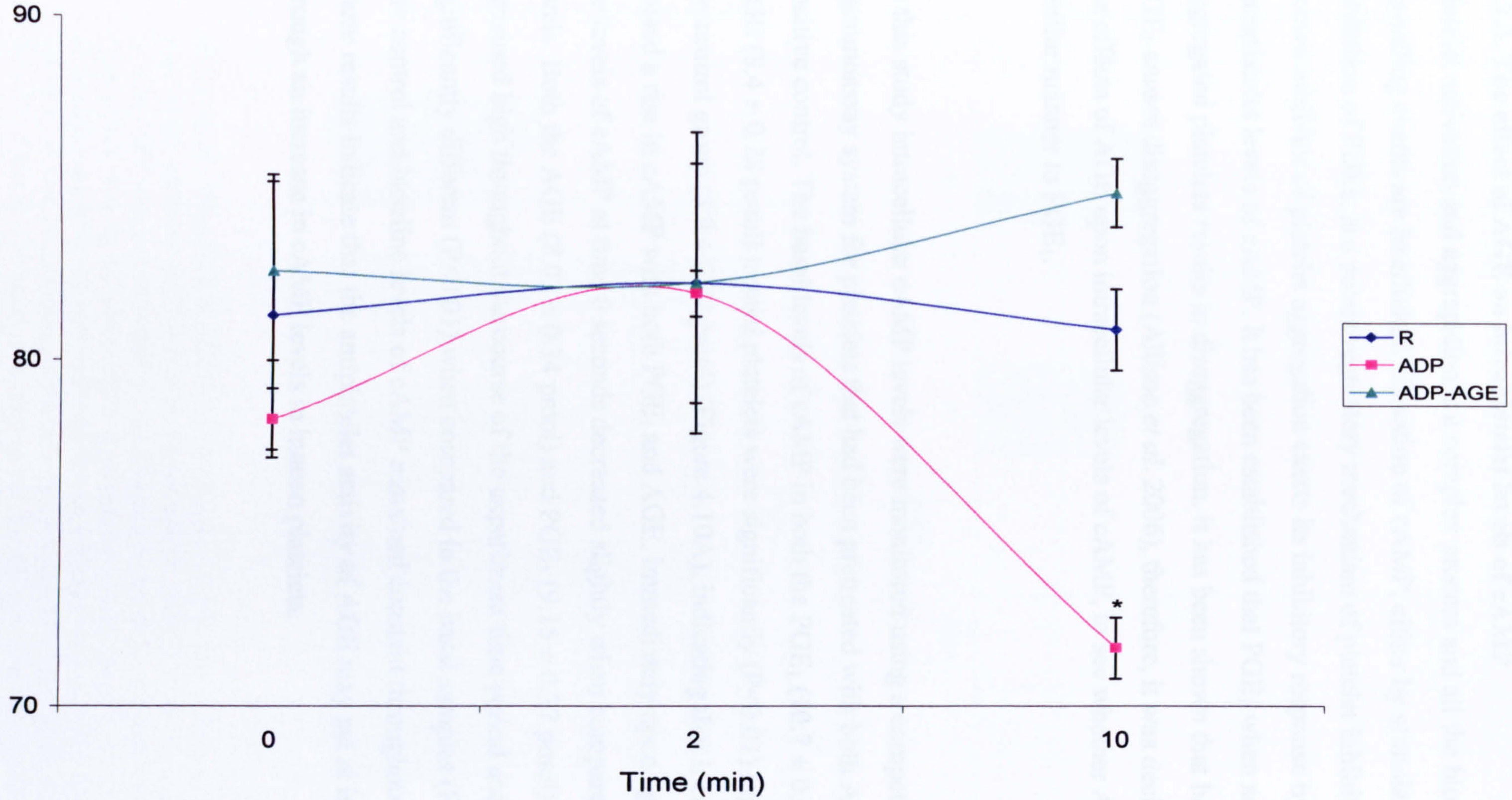


Figure 4.9. AA metabolism in both inactivated and activated platelets in the presence/absence of AGE. Platelet aggregation was initiated via the addition of either ADP (8 µmol/L) in the presence/absence of AGE (3.12 %, v/v) and the reaction terminated by placing aggregated platelets on ice. Platelets were washed and homogenised and treated with $CaCl_2$ and ^{14}C-AA + unlabelled sodium arachidonate (1 µCi + 1 µM/mL platelets, respectively). Aliquots were taken at set time points and extracted using ethyl acetate, dried and re-dissolved in ethanol. TLC plates spotted with samples and radioactivity measured using liquid scintillation. AA values calculated as a percentage of the total radioactive count (CPM) for that specific time point. *Statistically significant ($P<0.05$) when compared to the corresponding inactivated time sample (control), n=3. KEY: R – inactivated platelets; ADP – ADP activated platelets; ADP-AGE – AGE treated platelets activated with ADP.

4.2.3. The effect of AGE on intraplatelet levels of cAMP

Platelet activation and aggregation is a complex process and all the biochemical signalling events are interlinked. Elevation of cAMP, either by stimulation of AC or by inhibition of PDEs, is a potent regulatory mechanism of platelet inhibition. PGE_1, a known inhibitor of platelet aggregation exerts its inhibitory response by increasing the intraplatelet levels of cAMP. It has been established that PGE_1 when added to pre-aggregated platelets results in disaggregation. It has been shown that both AGE and PGE_1 causes disaggregation (Allison *et al.* 2006), therefore, it was decided to monitor the effects of AGE upon intracellular levels of cAMP, to see whether AGE acted in a similar manner to PGE_1.

In this study intracellular cAMP levels were monitored using a competitive enzyme immunoassay system for platelets that had been pretreated with both AGE or PGE_1, as a positive control. The basal levels of cAMP in both the PGE_1 (10.7 ± 0.42 pmol) and AGE (8.4 ± 0.28 pmol) treated platelets were significantly ($P<0.01$) higher than those of the control group (3.9 ± 0.72 pmol) (Figure 4.10A), indicating that incubation had caused a rise in cAMP with both PGE_1 and AGE. Immediately upon agonist stimulation the levels of cAMP at time 0 seconds decreased slightly when compared to the basal levels. Both the AGE (8.03 ± 0.34 pmol) and PGE_1 (9.15 ± 0.37 pmol) treated samples remained high throughout the course of the experiment time period and were significantly different ($P<0.01$) when compared to the basal samples (Figure 4.10B). The control and baseline levels of cAMP remained constant throughout the experiment. These results indicate that the antiplatelet activity of AGE may act at least partly through an increase in cAMP levels in human platelets.

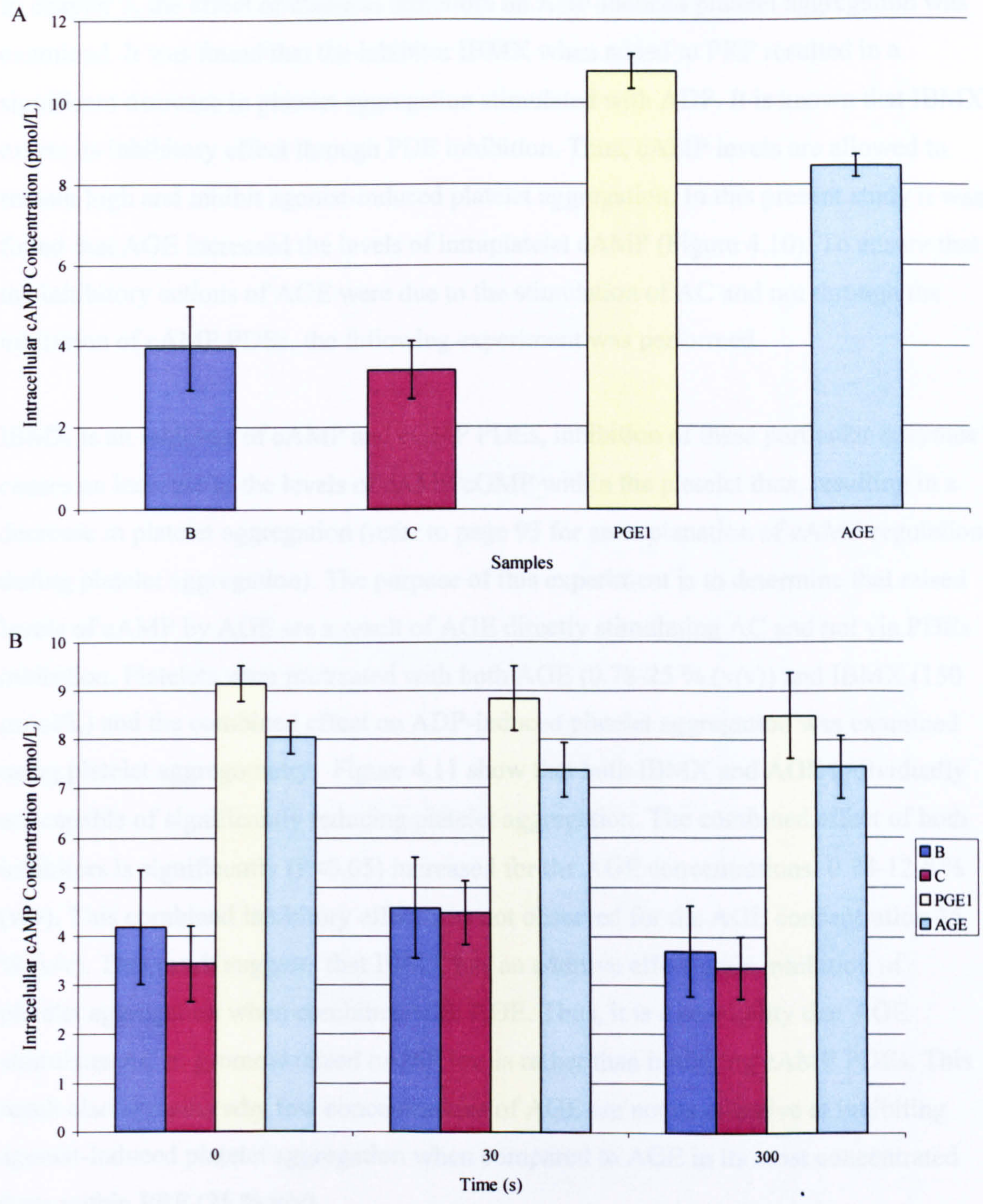


Figure 4.10. The effect of AGE on intraplatlet cAMP levels in both activated and inactivated platelets. AGE increases intraplatelet cAMP in ADP stimulated platelets (B) *in vitro*. PRP was pre-incubated in the absence or presence of either PGE_1 (10 μM) or AGE (25 % v/v) for 10 min at 37°C. Aggregation was initiated by the addition of ADP (8 μmol/L) and aliquots were taken before aggregation (A) and at the following time points 0, 30 and 300 seconds, at which point the platelets were immediately processed for cAMP levels. The assay was carried out in duplicate, and the results shown are means ± SEM, n=6. All values, both PGE_1 and AGE treated platelets are statistically significant ($P<0.01$) when compared to their respective control samples for both Figures A and B. KEY: B – basal, inactivated platelets; C – control, platelets treated with PBS, Figure A-unstimulated platelets, Figure B-ADP activated platelets; PGE_1 – platelets pretreated with PGE_1 and activated with ADP; AGE – AGE treated platelets activated with ADP.

In chapter 3, the effect of classical inhibitors on ADP-induced platelet aggregation was examined. It was found that the inhibitor IBMX when added to PRP resulted in a significant decrease in platelet aggregation stimulated with ADP. It is known that IBMX exerts its inhibitory effect through PDE inhibition. Thus, cAMP levels are allowed to remain high and inhibit agonist-induced platelet aggregation. In this present study it was found that AGE increased the levels of intraplatelet cAMP (Figure 4.10). To ensure that the inhibitory actions of AGE were due to the stimulation of AC and not through the inhibition of cAMP PDEs, the following experiment was performed.

IBMX is an inhibitor of cAMP and cGMP PDEs, inhibition of these particular enzymes causes an increase in the levels of cAMP/cGMP within the platelet thus, resulting in a decrease in platelet aggregation (refer to page 93 for an explanation of cAMP regulation during platelet aggregation). The purpose of this experiment is to determine that raised levels of cAMP by AGE are a result of AGE directly stimulating AC and not via PDEs inhibition. Platelets were pretreated with both AGE (0.78-25 % (v/v)) and IBMX (150 μmol/L) and the combined effect on ADP-induced platelet aggregation was examined using platelet aggregometry. Figure 4.11 show that both IBMX and AGE individually are capable of significantly reducing platelet aggregation. The combined effect of both inhibitors is significantly ($P<0.05$) increased for the AGE concentrations: 0.78-12.5 % (v/v). This combined inhibitory effect was not observed for the AGE concentration 25 % (v/v). This result suggests that IBMX has an additive effect upon inhibition of platelet aggregation when combined with AGE. Thus, it is a possibility that AGE stimulates AC to promote raised cAMP levels rather than inhibiting cAMP PDEs. This result also explains why low concentrations of AGE are not as effective at inhibiting agonist-induced platelet aggregation when compared to AGE in its most concentrated form within PRP (25 % v/v).

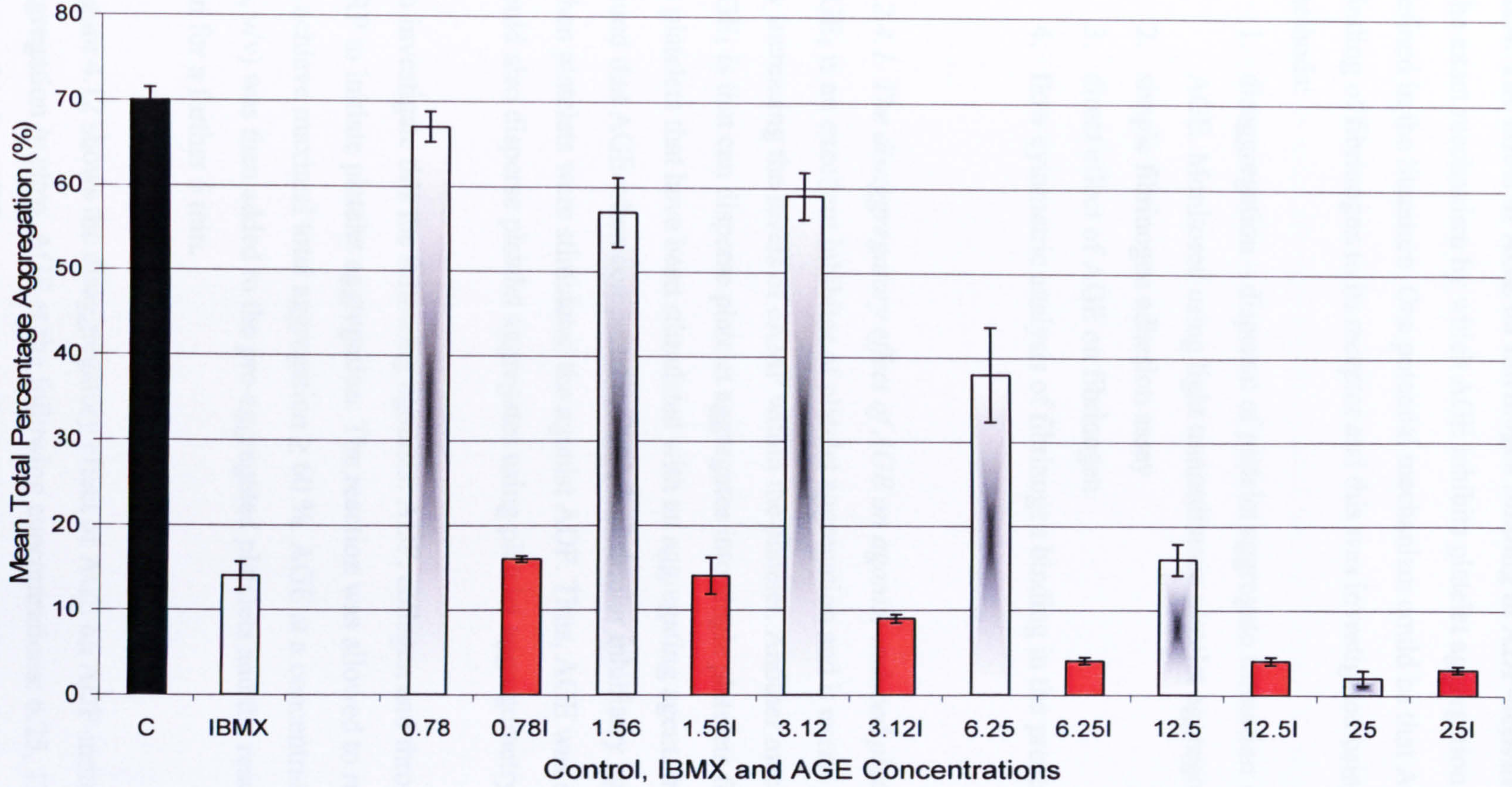


Figure 4.11. The combined effect of AGE and IBMX on ADP-induced platelet aggregation. IBMX (150 µmol/L) and AGE (0.78-25 %, v/v) were pre-incubated with PRP both alone and as a mixture. Aggregation was initiated with ADP (8 µmol/L). Aggregation curves were analysed for mean total percentage aggregation and values are expressed as means ± SEM, n=6. All the IBMX + AGE samples were statistically significant ($P<0.05$) when compared to both the control and corresponding AGE concentration (except AGE 25 %). Shaded bars represent AGE on its own; red bars indicate IBMX + AGE.

4.2.4. The effect of AGE on fibrinogen binding in ADP-activated platelets

The exact mechanism by which AGE inhibits platelet aggregation has not been clearly defined in the literature. One potential mechanism could be that AGE interferes with the binding of fibrinogen to its receptor and this was investigated using four independent methods:

1. disaggregation – dispersal of platelet aggregate formation via the addition of AGE. Monitored using light transmittance platelet aggregometry
2. simple fibrinogen adhesion assay
3. direct effect of AGE on fibrinogen
4. flow cytometric analysis of fibrinogen binding in the presence/absence of AGE

4.2.4.1. The disaggregatory effect of AGE on agonist-induced platelet aggregation

PGE_1 is an excellent inhibitor of platelet aggregation and it exerts its inhibitory effect by increasing the levels of cAMP within the platelet. Another mechanistic action of PGE_1 is that can disperse platelet aggregates into single platelets following its addition to platelets that have been stimulated with an aggregating agent. In chapter 3, it was found that AGE when compared to PGE_1 had similar inhibitory aggregation profiles when platelets were stimulated the agonist ADP. Thus, AGE was examined to see if it could also disperse platelet aggregates using platelet aggregometry.

To investigate this the following agonists: ADP, collagen and thrombin were added to PRP to initiate platelet aggregation. The reaction was allowed to run for 5 min in order to achieve maximal total aggregation $\geq$ 60 %. AGE at a concentration range of (0.78-25 %, v/v) was then added to the pre-aggregated platelets and the reaction was allowed to run for a further 5 min.

Figure 4.12 shows the disaggregatory effect of AGE on ADP-induced platelet aggregation *in vitro*. AGE at the following concentrations: 6.25, 12.5 and 25 % (v/v) when added to ADP pre-aggregated platelets resulted in significant ($P<0.05$) disaggregation (dispersal of a platelet aggregate) furthermore. At the AGE concentration 25 % (v/v) there was complete disaggregation (100 %). Collagen and thrombin were also tested, as these are the main agonists used in the initiation of platelet aggregation *in vivo.* Figures 4.13 and 4.14 depict the disaggregatory effects of AGE on

platelet aggregation initiated with collagen and thrombin, respectively. AGE at the concentrations 3.12-25 % (v/v) when added to platelets that had been pre-aggregated with collagen resulted in significant disaggregation ($P<0.05$). Platelets aggregated with thrombin showed that AGE (1.56-25 %, v/v) significantly ($P<0.05$) reversed platelet aggregation when compared to the control sample. However, complete disaggregation was only noted in platelets that had been preaggregated with the agonist ADP.

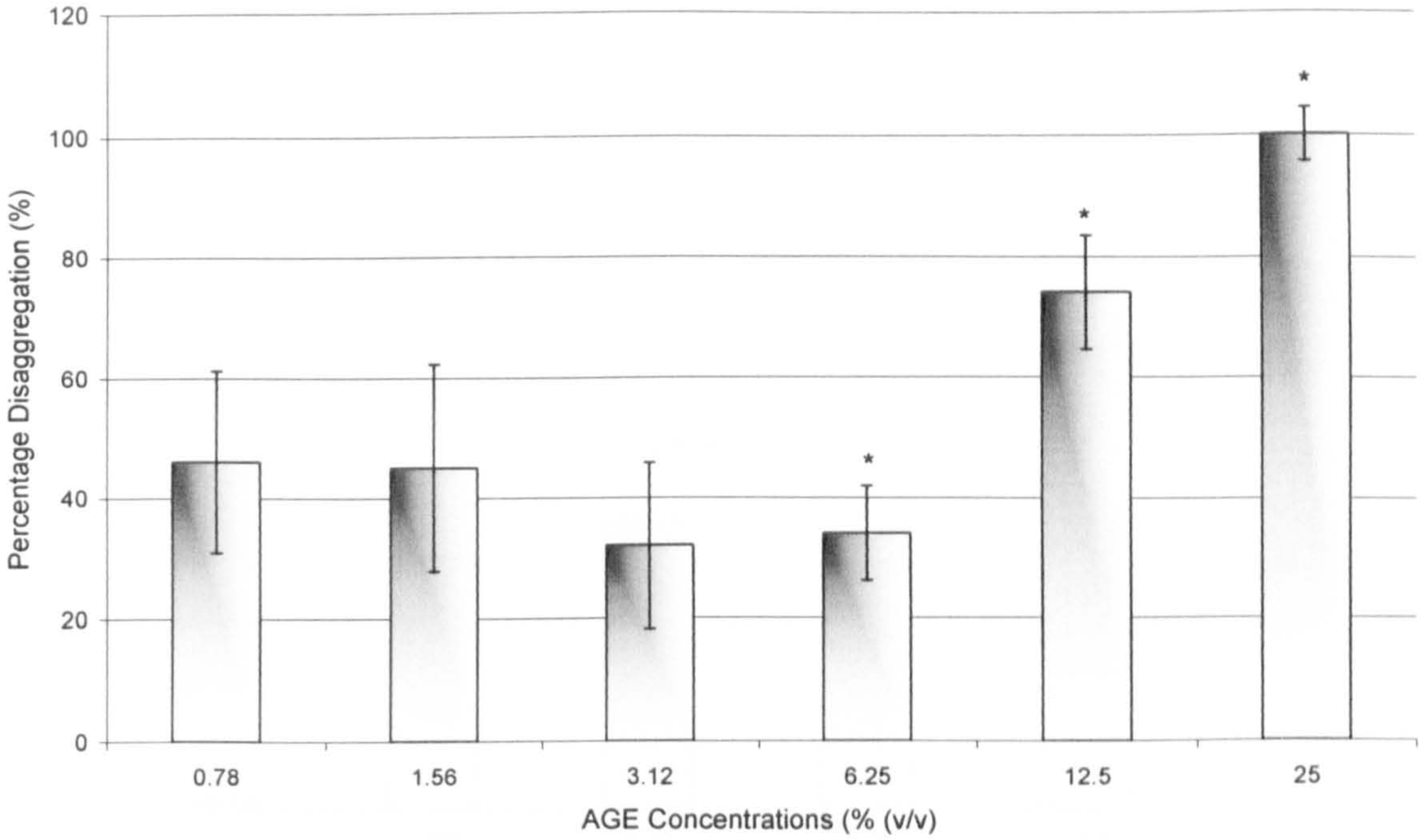


Figure 4.12. The disaggregatory effect of AGE at various final percentage concentrations (%) on platelet aggregation initiated by ADP *in vitro*. Values are means ± SEM, n=6. Values are expressed as a percentage of the amount of disaggregation produced by the AGE concentration compared to the PBS control (62 ± 4.5) *Statistically significant ($P<0.05$) compared to the control.

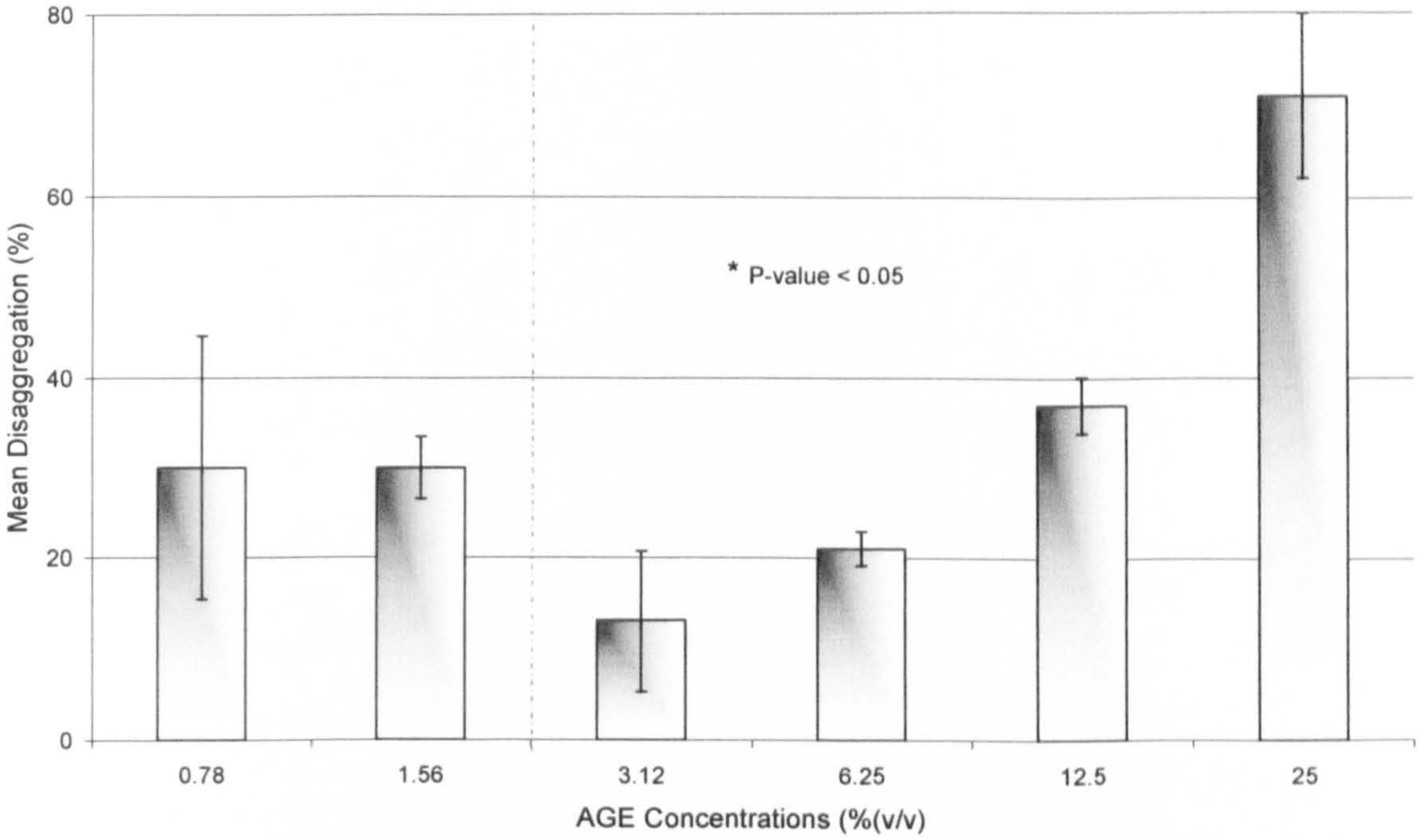


Figure 4.13. The disaggregatory effect of AGE at various final percentage concentrations (%) on platelet aggregation initiated by collagen *in vitro*. Values are means ± SEM, n=6. Values are expressed as a percentage of the amount of disaggregation produced by the AGE concentration compared to the PBS control (86 ± 5.3) *Statistically significance ($P<0.05$) was observed for the AGE concentrations (3.12-25 %, v/v) compared to the control this is indicated on the graph by a red line.

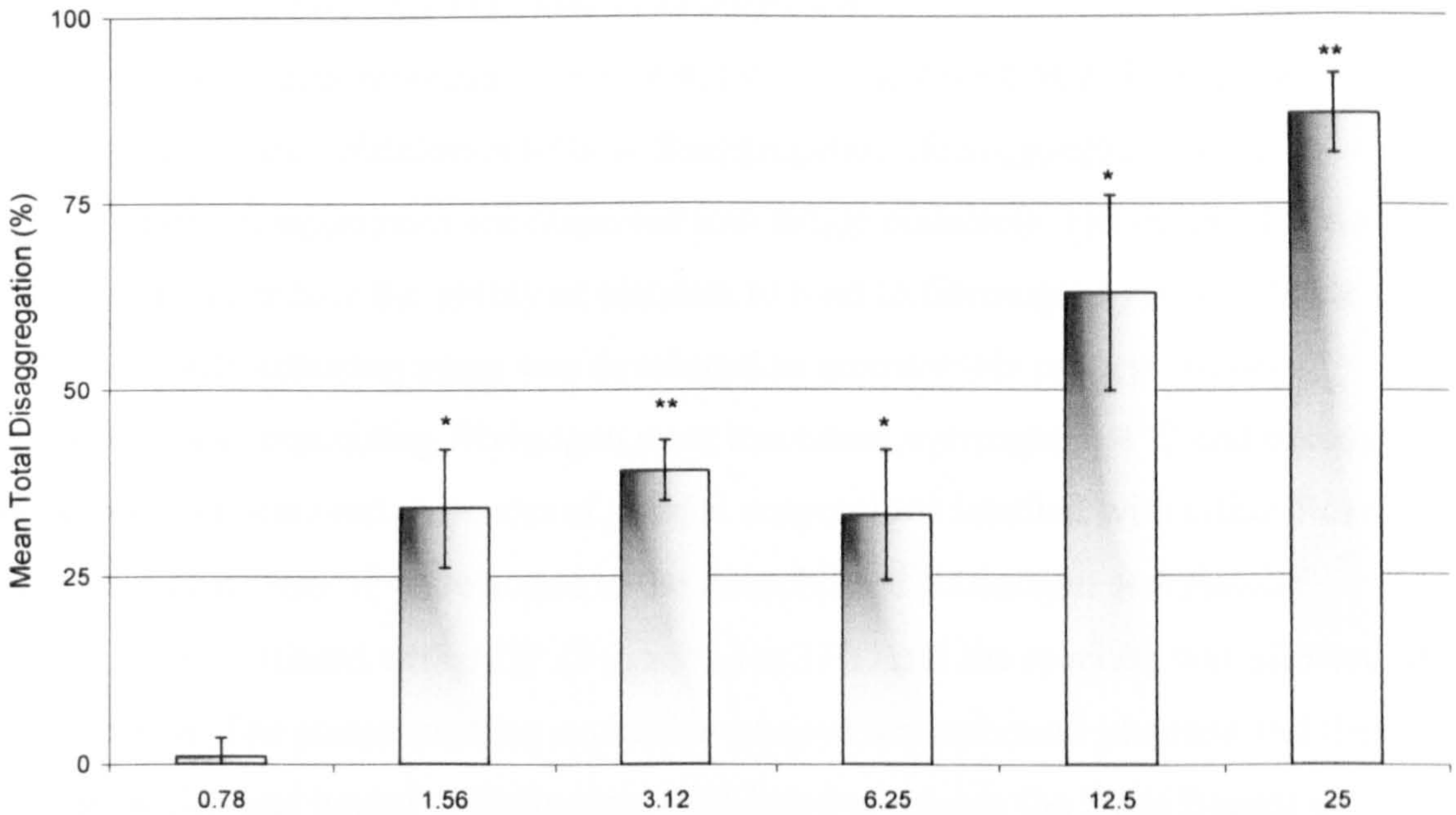


Figure 4.14. The disaggregatory effect of AGE at various final percentage concentrations (%) on platelet aggregation initiated by thrombin *in vitro*. Values are means ± SEM, n=6. Values are expressed as a percentage of the amount of disaggregation produced by the AGE concentration compared to the PBS control (84 ± 4.3) *Statistically significant ($P<0.05$), **($P<0.01$) compared to the control.

4.2.4.2. Simple adhesion assay for the measurement of the effect of AGE on fibrinogen binding during agonist-induced platelet aggregation

From the experiments reported in section 4.2.4.1 it is known that AGE when added to ADP pre-aggregated platelets results in disaggregation (the aggregation process is reversed, platelet aggregates are dispersed into single platelets). The mode of action of AGE may be to reduce the ability of platelets to bind to fibrinogen. To investigate this further a simple adhesion assay was developed to monitor this process. In brief, microtitre plates containing fibrinogen were incubated overnight at 4°C and excess fibrinogen was removed. Aliquots of platelet suspensions labelled with either Rose Bengal or chromium-51 were added to the immobilised fibrinogen and platelet activation was initiated with ADP (8 μmol/L) at 37°C and the reaction was allowed to run for 5 min. The plates are then washed to remove any unbound platelets and the platelets which had bound to fibrinogen were lysed to release the Rose Bengal or chromium-51, the optical density or radioactive counts were compared to a series of control samples to determine the amount of platelet adhesion.

Optical density or radioactivity counts represent the amount of platelets bound to the immobilised fibrinogen matrix through the release of Rose Bengal and chromium-51, respectively from the platelet cytosol. Figures 4.15 and 4.16 show that AGE does reduce the amount of adherent platelets to immobilised fibrinogen. A significant ($P<0.05$) reduction in fibrinogen binding was noted in the Rose Bengal treated platelets for the AGE concentrations: 3.12-12.5 % (v/v) when compared to the control. Platelets treated with chromium-51 also showed significant ($P<0.05$) reduction in platelet adhesion when pretreated with the AGE concentrations: 1.56, 6.25-25 % (v/v).

4.2.4.3. The direct effect of AGE on the fibrinogen molecule

In order to investigate whether AGE was having an effect at the fibrinogen receptor, the direct effect of AGE on the fibrinogen molecule was measured using the following experimental protocol:

A 96-well microtitre plate was coated with fibrinogen (3 g/L) and left overnight at 4°C. Various concentrations of AGE (0.78-25 % v/v) were added to the wells and left for 30 min at 37°C. The wells were then washed with 3x (200 μL) with PBS. PRP was added

to the wells and aggregation was initiated via the addition of ADP (8 μmol/L). This was followed by Rose Bengal for a further 30 min. The non-adherent platelets were removed via aspiration and the wells were washed with PBS. Finally, lysis buffer was added to the wells and the optical density at 540 nm was measured spectrophotometrically.

Fibrinogen (3 g/L) when pre-incubated with AGE (0.78-25 %, v/v) resulted in significant inhibition of platelet adhesion to platelets that had been activated with the agonist ADP (Figure 4.17). No trend was seen between any of the AGE concentrations used (no dose-dependency). Although an inhibitory effect is apparent from the results, the method is not sensitive enough to draw a valid conclusion from. Hence, more investigation is required in the form of flow cytometric analysis to determine if AGE is having a definite effect upon fibrinogen binding and the formation of platelet aggregates.

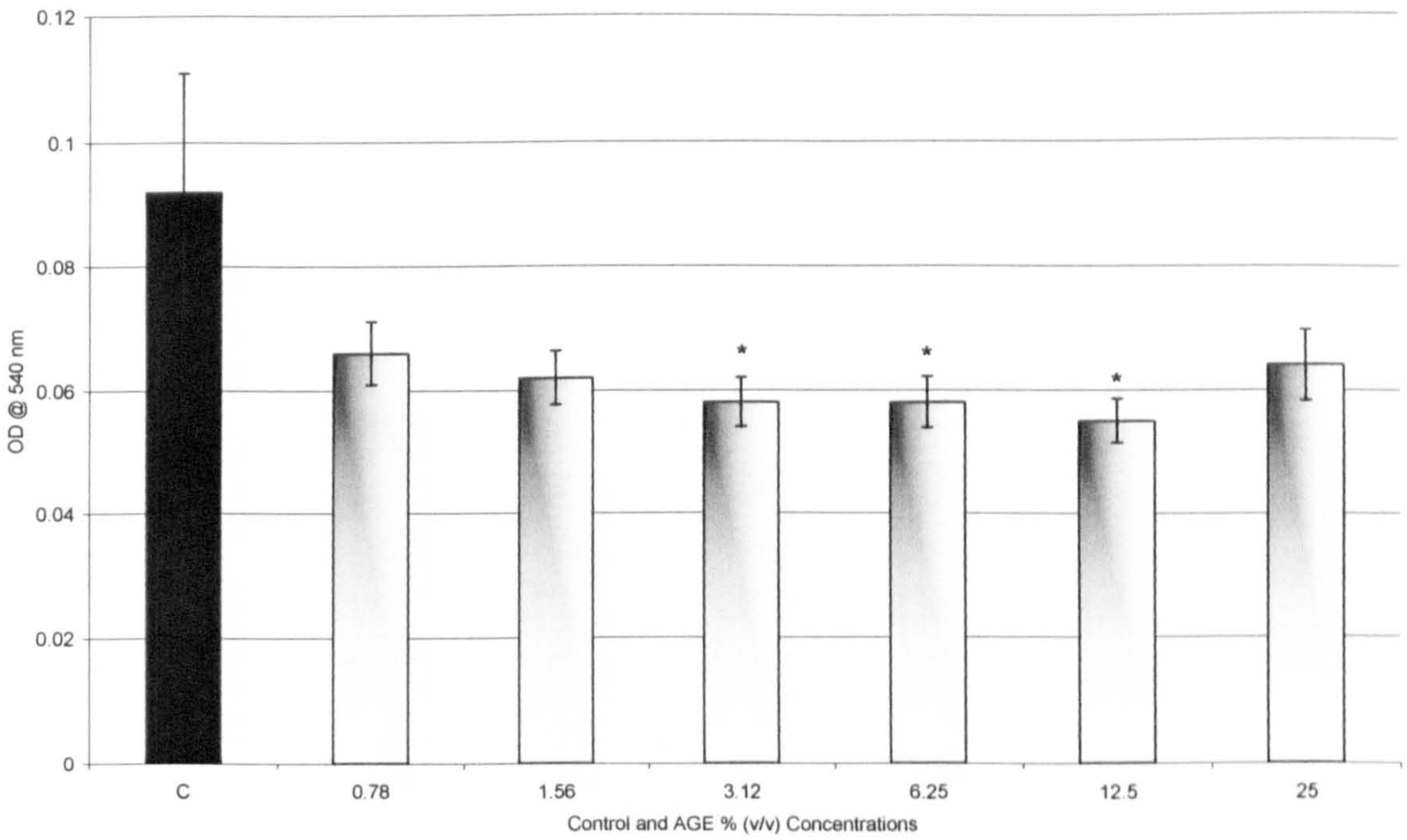


Figure 4.15. The effect of AGE at various concentrations on the ability of ADP-activated platelets labelled with Rose Bengal to bind to immobilised fibrinogen. PRP was incubated with various AGE concentrations (0.78-25 %, v/v) for 10 min at 37°C. AGE treated platelets were allowed to adhere to immobilised fibrinogen (3 g/L) via activation with ADP (8 μmol/L). Rose Bengal was added to the platelets and its release was measured spectrophotometrically at 540 nm as an indication of platelet adhesion. Values are expressed as means ± SEM, n=6. *Statistically significant ($P<0.05$) when compared to the PBS control.

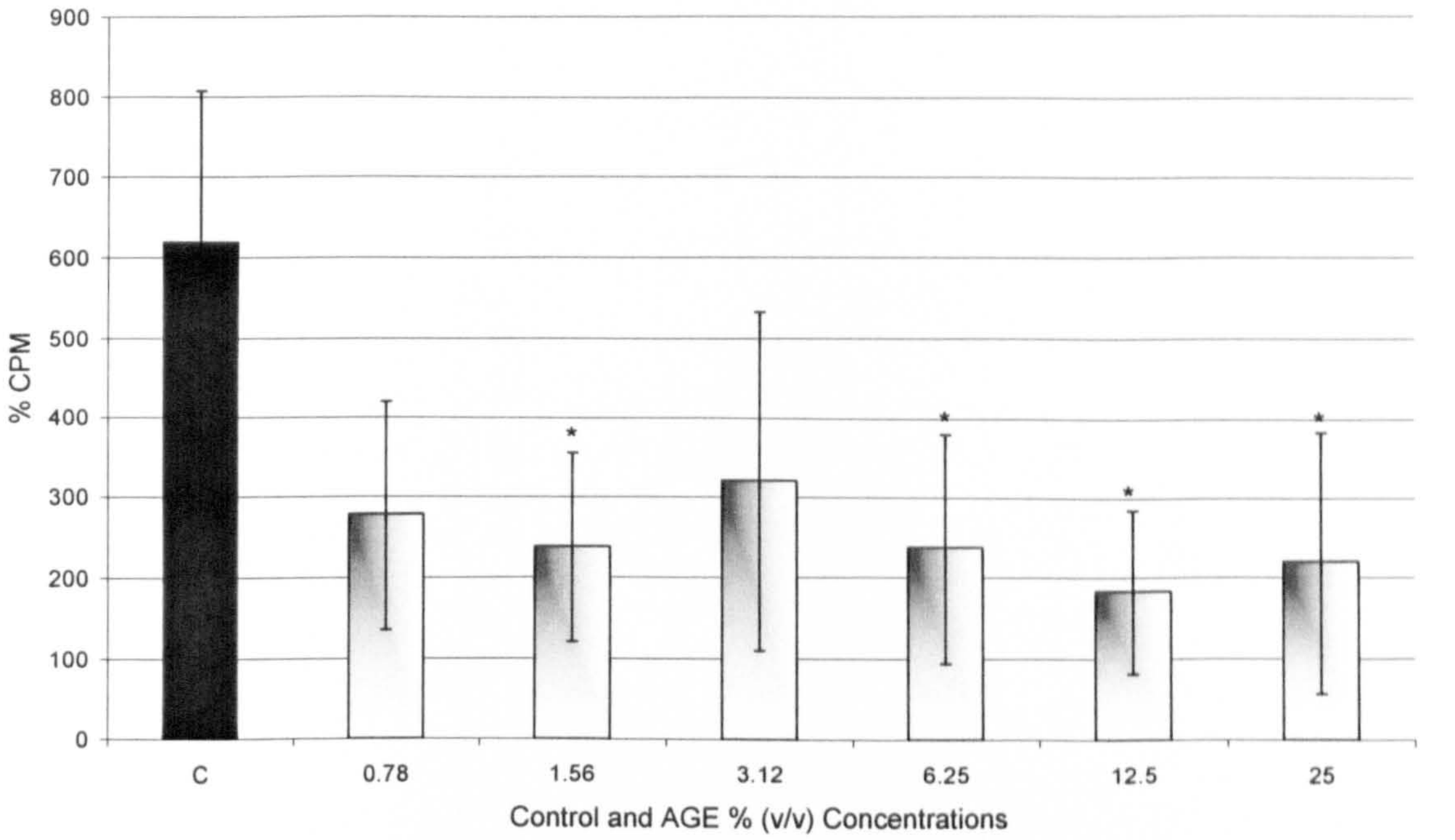


Figure 4.16. The effect of AGE at various concentrations on the ability of ADP-activated platelets labelled with chromium-51 to bind to immobilised fibrinogen. PRP was incubated with various AGE concentrations (0.78-25 %, v/v) for 10 min at 37°C. AGE treated platelets were allowed to adhere to immobilised fibrinogen (3 g/L) via activation with ADP (8 μmol/L). Chromium-51 was added to the platelets and its release was measured via liquid scintillation as an indication of platelet adhesion. Values are expressed as means ± SEM, n=6. *Statistically significant ($P<0.05$) when compared to the PBS control.

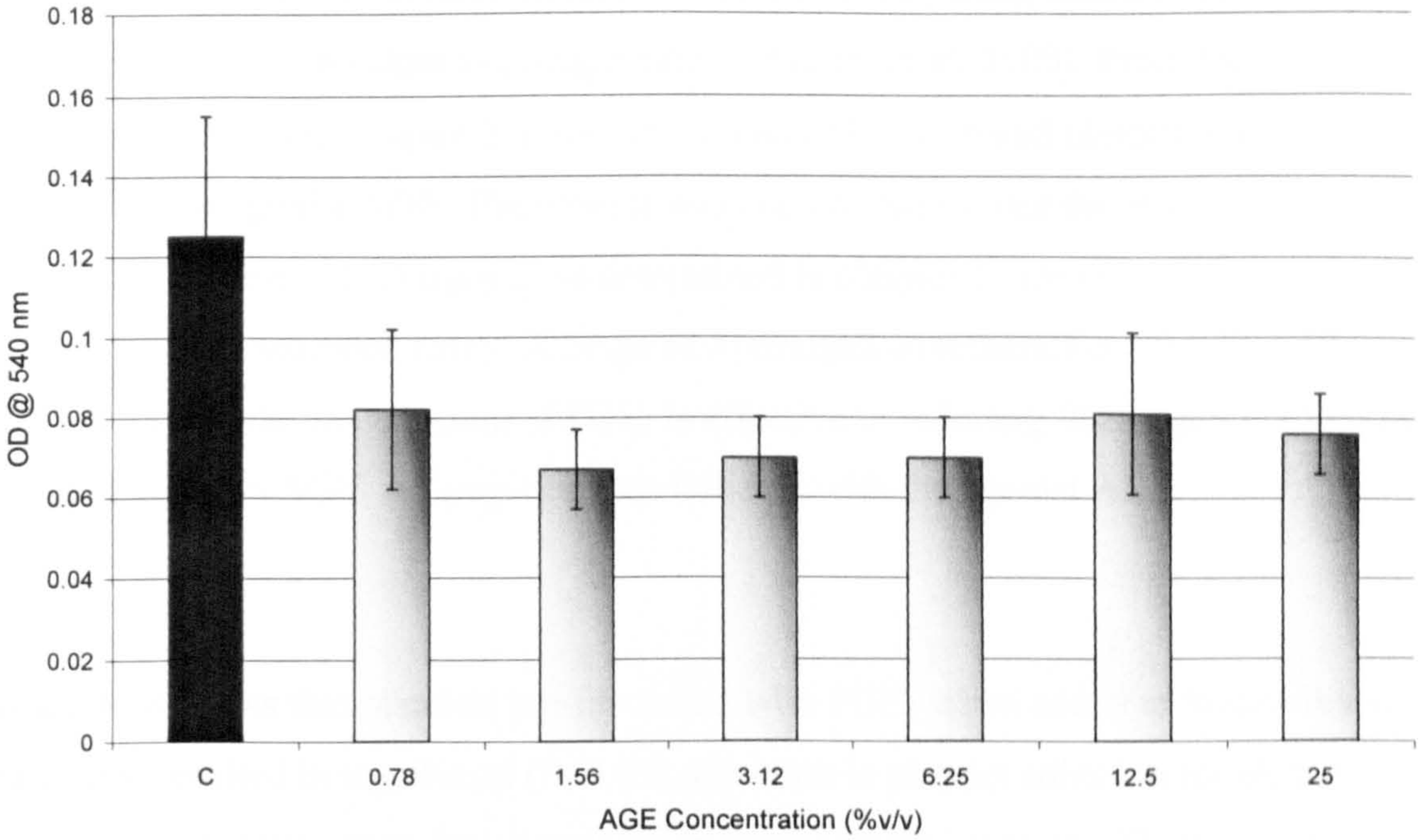


Figure 4.17. The direct effect of AGE on fibrinogen. Fibrinogen (3 g/L) was incubated with AGE (0.78-25 %, v/v) for 10 min at 37°C. PRP was added to the AGE-coated fibrinogen and aggregation was initiated with ADP (8 μmol/L). Rose Bengal was added to the adherent platelet, lysed and measured spectrophotometrically at 540 nm. Values are means ± SEM, n=6. All the AGE concentrations significantly ($P<0.05$) reduced platelet adhesion compared to the control.

These experiments were conducted on the basis that PGE_1 when added to pre-aggregated platelets resulted in disaggregation (Kikura *et al.* 2000). From the aggregation studies in chapter 3, it was found that PGE_1 inhibited platelet aggregation induced by the agonist ADP. Therefore it was decided to monitor the effects of PGE_1 using its IC_{50} value (31.25 μg/mL) as determined in chapter 3. This was achieved by using the simple adhesion assay. A range of fibrinogen concentrations (0.003-3 g/L) were tested in order to determine if PGE_1 is effective in reducing fibrinogen binding in a similar manner to AGE. Aggregation was initiated with the agonist ADP.

Figure 4.18 shows that platelets pre-incubated with PGE_1 when added to immobilised fibrinogen resulted in significant ($P<0.05$) reduction in platelet adhesion for all the fibrinogen concentrations when compared to the respective controls. There was no difference in the actions of PGE_1 for each of the fibrinogen concentrations examined.

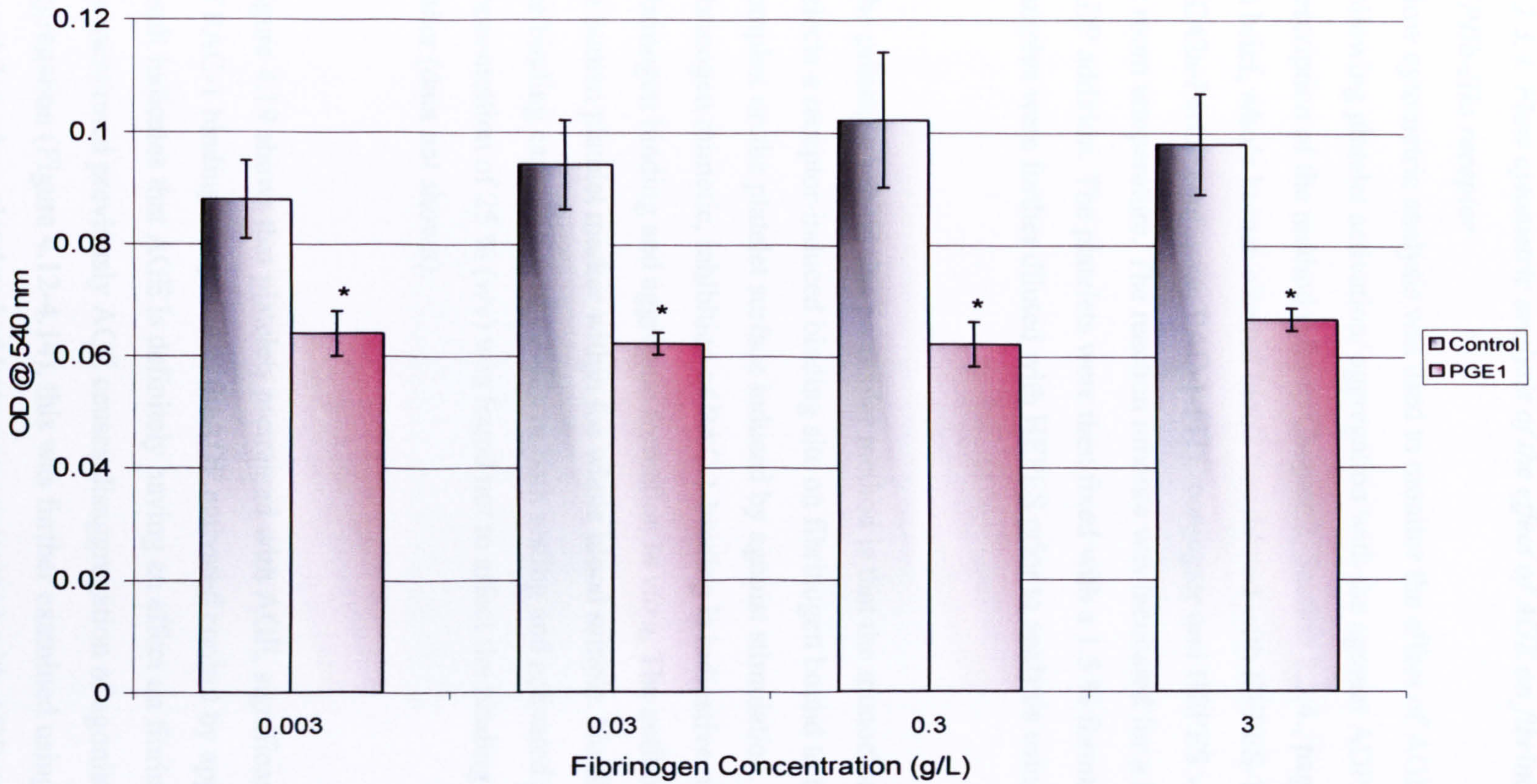


Figure 4.18. The effect of PGE_1 (31.25 µg/mL) on the ability of ADP-activated platelets labelled with Rose Bengal to bind to various concentrations of immobilised fibrinogen (0.000-3.0 g/L). PRP was incubated with PGE_1 for 10 min at 37°C. PGE_1 treated platelets were allowed to adhere to immobilised fibrinogen (0.003-3 g/L) via activation with ADP (8 µmol/L). Rose Bengal was added to the platelets and its release was measured spectrophotometrically at 540 nm as an indication of platelet adhesion. Values are expressed as means ± SEM, n=6. *Statistically significant ($P<0.05$) when compared to the respective PBS control.

4.2.3.4. Flow cytometric analysis of the effect of AGE on fibrinogen binding to the GPIIb-IIIa receptor

Flow cytometric analysis was used to monitor the effect of AGE on fibrinogen binding following platelet activation/ aggregation with the agonist ADP. For a detailed description of the method refer to Chapter 2, Section 2.5.4., page 44.

In brief, whole human citrated blood was diluted with HEPES-Tyrode's buffer. AGE, CD42a-PerCP conjugate, PAC-1-FITC conjugate and HEPES was incubated for 10 min at room temperature. The reaction mixture was incubated for a further 15 min following ADP addition. The platelets were then fixed with a 1.5 % formaldehyde solution and the samples were further diluted with HEPES prior to analysis using the flow cytometer.

The principle behind this particular method is that the monoclonal antibody PAC-1 detects a receptor-induced binding site on fibrinogen bound to the GPIIb/IIIa receptor complex on the platelet surface induced by agonist stimulation. As PAC-1 is a fibrinogen mimetic, inhibition of PAC-1 binding is indicative of a reduction in fibrinogen binding and aggregate formation *in vitro*. The antibody CD42a was used as an human platelet marker within the whole blood sample. No difference was noted in the binding capabilities of CD42a in both resting and activated platelets, AGE at a final concentration of 25 % (v/v) was found not to affect the binding capabilities of CD42a either (data not shown).

Figure 4.19 shows that platelets pretreated with AGE, significantly inhibited the amount of PAC-1 binding compared to theADP activated control by approximately 50 %. This result indicates that AGE is definitely having an effect on fibrinogen binding in vitro. As mentioned previously AGE causes disaggregation of agonist-induced platelet aggregation (Figure 4.12-4.14), this was further examined using flow cytometry. It was found that when platelets had been pre-aggregated with ADP and AGE added to the aggregating platelets; AGE caused disaggregation by reducing the amount of bound PAC-1 by 40 % when compared to the control sample as shown in Figure 4.17. This study supports the findings from the disaggregation and fibrinogen adhesion assay experiments, that AGE inhibits platelet aggregation by affecting the binding properties of fibrinogen to its receptor GPIIb/IIIa.

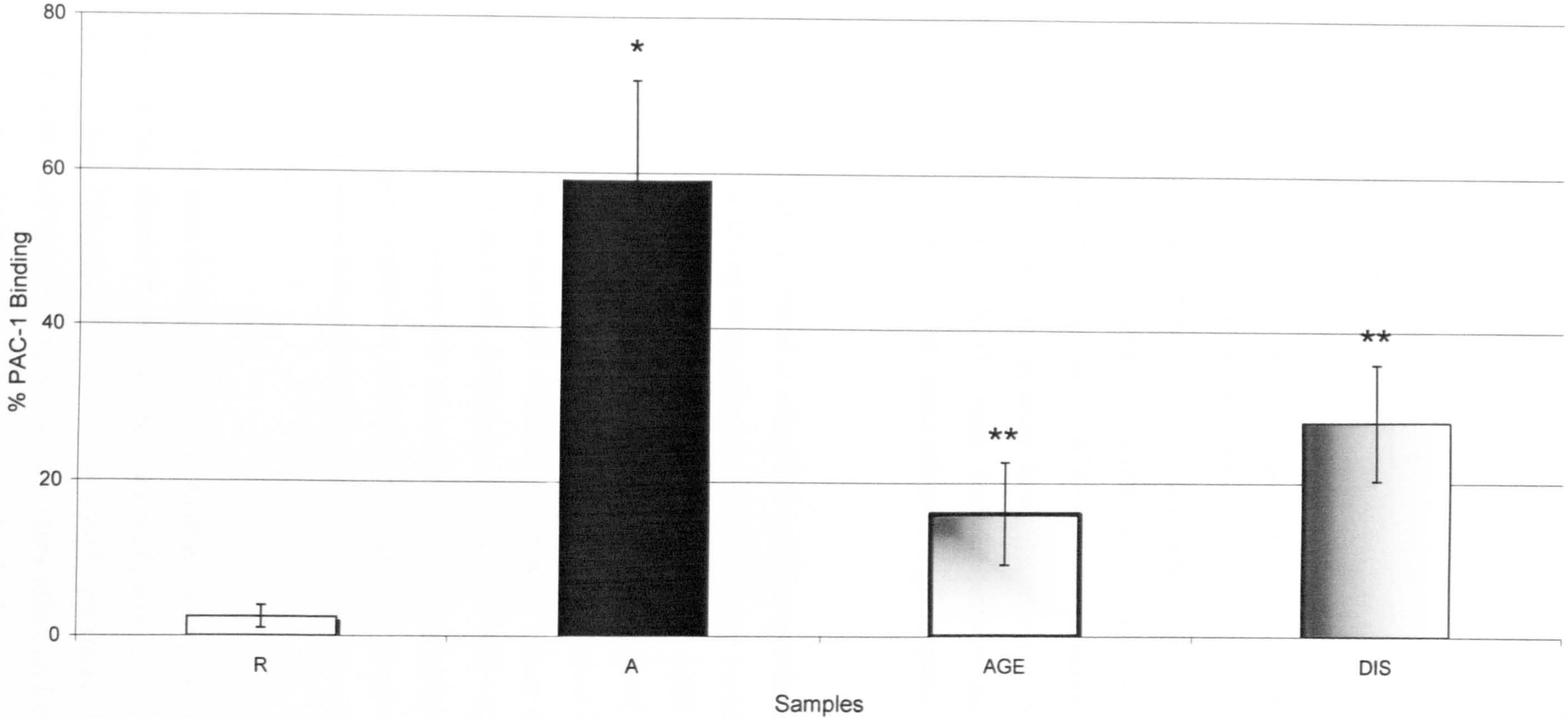


Figure 4.19. Flow cytometric analysis of the inhibitory effect of AGE on fibrinogen binding to its receptor GPIIb-IIIa. GPIIb-IIIa receptor binding was monitored using the antibody PAC-1 FITC as a marker in whole citrated blood. Values are expressed as means ± SEM, n=4. *Statistically significant ($P<0.05$), **($P<0.01$) compared to the control. KEY: R – inactivated platelets; A – ADP activated platelets; AGE – AGE treated platelets activated with ADP; DIS – platelet disaggregation, AGE is added to ADP activated platelets.

4.3. IMAGING STUDIES TO MONITOR THE EFFECT OF AGE ON CALCIUM MOBILISATION AND FIBRINOGEN BINDING

Fluorescence microscopy and SEM were used to capture images of the effects of AGE on both calcium mobilisation and fibrinogen binding during ADP-induced platelet aggregation. In this preliminary study it was found that AGE may inhibit platelet aggregation by suppressing the mobilisation of calcium during agonist stimulation. Using fluorescence microscopy the aim was to show that in the presence of AGE the intensity of the fluorescence signal emitted by Fura-2 is less than that of the control platelets. The images presented in figures 4.20A and 4.20B show that there is a decrease (see Figure 4.20B) in the intensity of fluorescence in platelets treated with AGE and activated with ADP when compared to ADP-activated platelets (Figure 4.20A). The figures show that there is more aggregate formation on the control slide compared to platelets pretreated with AGE. This result further support evidence presented in this study that AGE may exert its inhibitory effect through suppression of calcium mobilisation.

In this study AGE also appeared to affect the binding capabilities of the fibrinogen receptor GPIIb-IIIa. Using SEM, the aim of this particular experiment was to show that platelet aggregate formation is inhibited in the presence of AGE. Figures 4.21A-C show the images obtained from the SEM, figure 4.21A is an image of platelets in a resting/inactivated state and, they are discoid in shape. Figure 4.21B, represents platelets that have been activated with the agonist ADP. Figure 4.21C depicts platelets that have been pre-treated with AGE and activated with ADP. It is not very clear from the images as the microscope was not capable of examining platelets in great detail, but, the captured images suggest that AGE is having an effect on platelet aggregation, in that AGE treated platelets displayed less aggregate formation when compared to untreated platelets (control).

It is also apparent from the experiment that AGE allows shape change to occur (platelets go from a resting discoid shape to the formation of pseudopia) however, aggregation is not as prominent. This reinforces the previous work (fibrinogen binding study) suggesting that AGE is having a possible inhibitory effect on fibrinogen binding to its receptor and the subsequent formation of a stable platelet aggregate.

However, it should be noted this investigation was a preliminary one and further work needs to be conducted to ensure that the observed results are a true indication of calcium mobilisation suppression and a reduction in fibrinogen binding, respectively. For the purpose of this study the images do suggest that calcium mobilisation and fibrinogen binding is being reduced in the presence of AGE when compared to their respective controls.

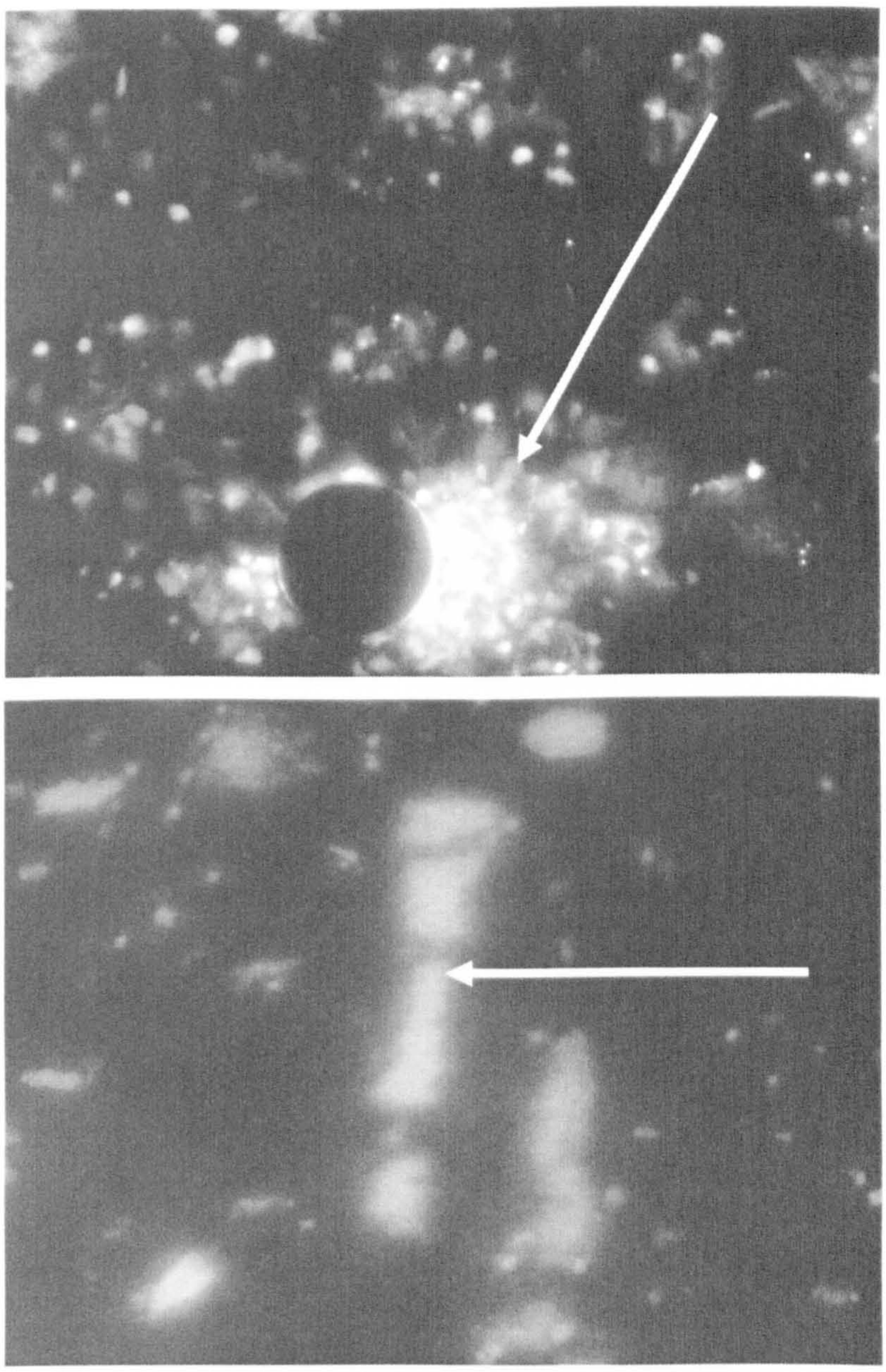
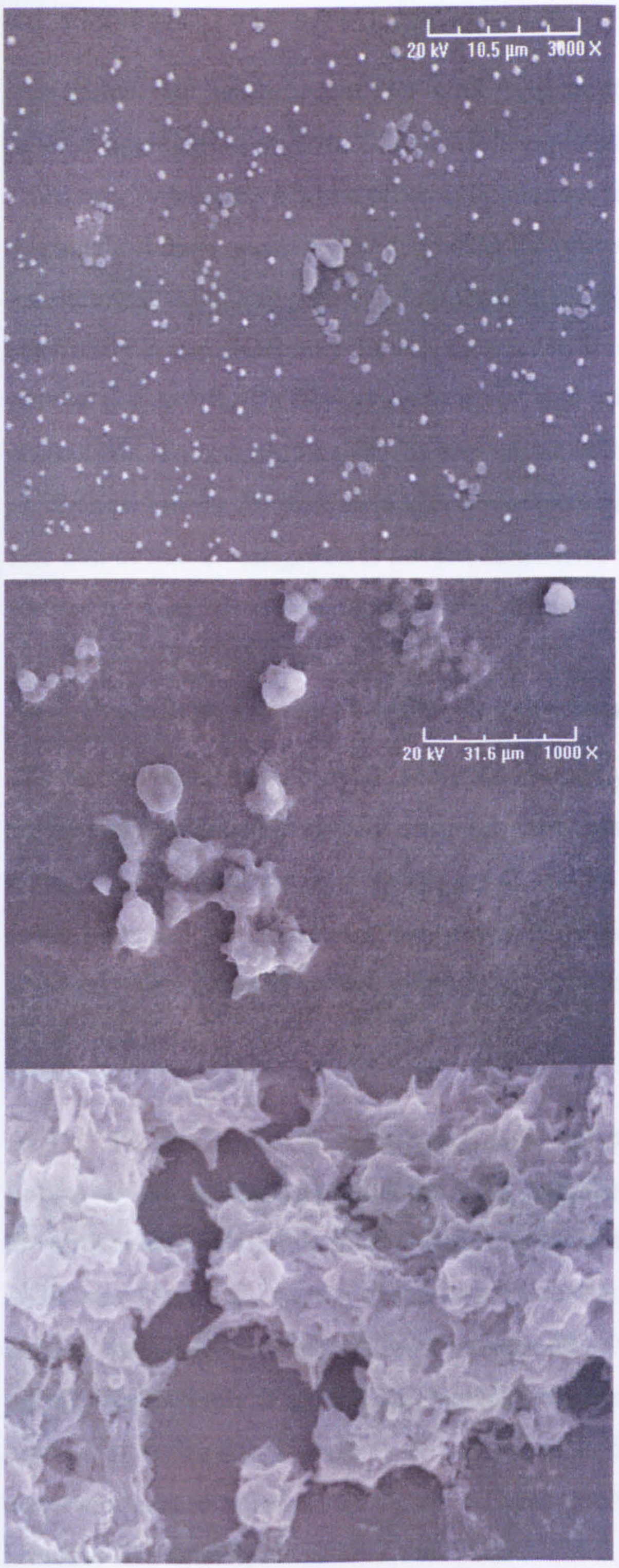


Figure 4.20. Fluorescence microscopy imaging of the effect of AGE on calcium mobilisation using the calcium indicator dye Fura-2/AM. The effect of AGE on calcium mobilisation was monitored using fluorescence spectrophotometry. Platelets were loaded with the calcium indicator dye Fura-2/AM and were activated with ADP. The activated platelets were allowed to adhere to poly-$_L$-lysine coated coverslips. Key: A – ADP activated platelets in the absence of AGE (control); B – ADP activated platelets in the presence of AGE. White arrows indicate platelet aggregate formation.



A

B

C

Figure 4.21. SEM imaging of the effect of AGE on the ability of platelets to form aggregates when challenged with the agonist ADP. Key: A – platelets at rest/unactivated; B – ADP-induced platelet aggregation; C – AGE treated platelets, aggregation initiated with ADP.

4.4. PROTEOLYTIC ACTIVITY OF AGE

It is known that incubation of PRP with AGE results in the inhibition of agonist-induced platelet aggregation *in vitro*. One possible explanation for the observed inhibition is that AGE may potentially act in a proteolytic manner thus, preventing platelet activation and aggregation from occurring by degrading the proteins (e.g. receptors) needed for the biochemical signalling pathways involved in the aggregatory process. Another possibility is that AGE may directly interact with receptors located on the surface of the platelet (i.e. GPIIb/IIIa fibrinogen receptor) and prevent normal function through proteolysis. To investigate this, the proteolytic activity of AGE was measured using two independent methods: azocasein spectrophotometric assay and SDS-PAGE analysis. The aim of this investigation is not to characterise the individual proteases that may be present in AGE but to determine if AGE displays proteolytic activity.

4.4.1. Proteolytic activity of AGE using the Azocasein spectrophotometric assay

The proteolytic activity of AGE was determined by using the azocasein assay, which monitors proteolytic activity by comparing the effects of potential protease(s) contained within AGE to the actions of chymotrypsin and its substrate casein. The assay works colourimetrically by measuring the release of casein from the azo group to which it is attached. For a detailed method of the assay see Chapter 2 (Materials and Methods, page 47).

AGE (3.12-100 %, v/v) was incubated for 30 min at 37° with the substrate azocasein. Following the incubation period, the AGE samples underwent TCA precipitation and the optical densities (in duplicate) were measured at 450 nm. The proteolytic activity of AGE was determined from a chymotrypsin standard curve. Proteolytic activity is evident for the AGE concentrations between 50 - 100 % (v/v) and was 32 and 118 µg/mL, respectively (Figure 4.22). Values were obtained from a chymotrypsin standard curve (Appendix III). There was no proteolytic activity observed for the remaining AGE concentrations tested (3.12-25 %, v/v). These results suggest that AGE possesses protease-like activity.

Chymotrypsin is specific for the substrate casein, and although proteolytic activity is evident in the AGE samples, 50 and 100 % (v/v), the results are not a true indication of

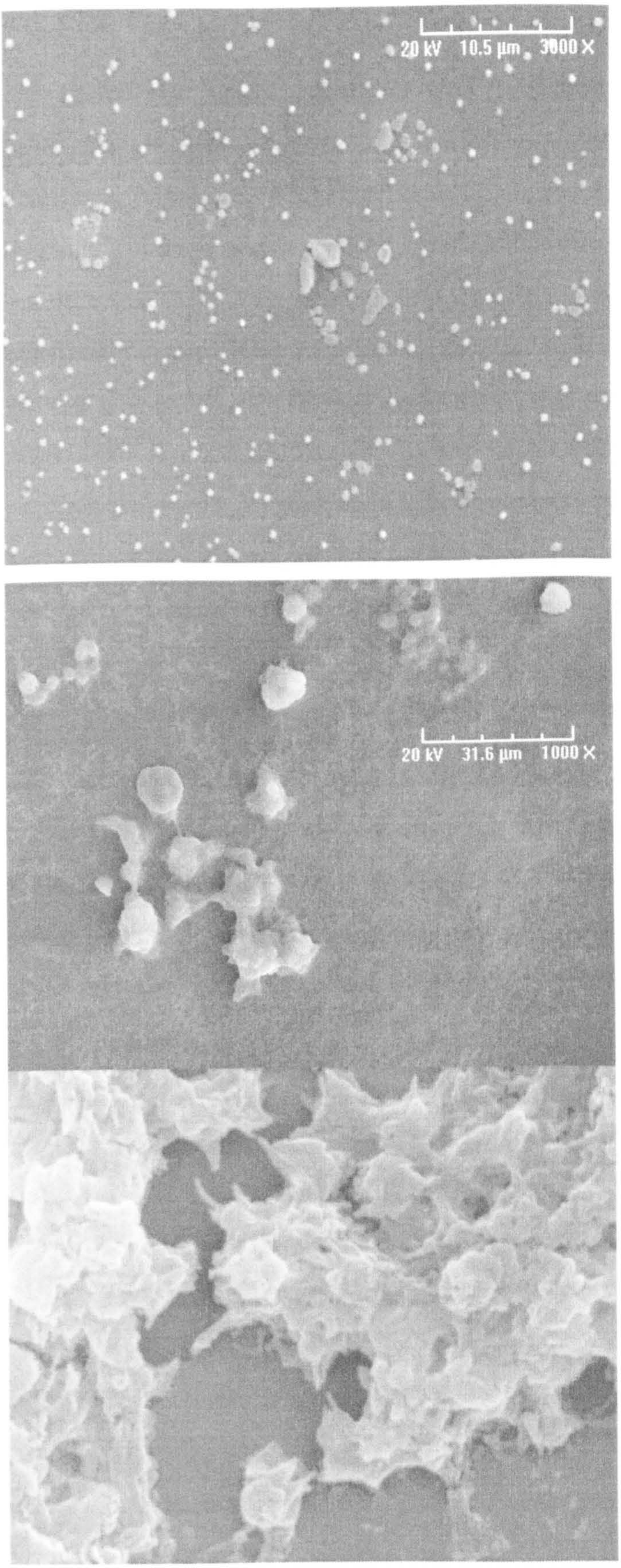


Figure 4.21. SEM imaging of the effect of AGE on the ability of platelets to form aggregates when challenged with the agonist ADP. Key: A – platelets at rest/unactivated; B – ADP-induced platelet aggregation; C – AGE treated platelets, aggregation initiated with ADP.

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Chymotrypsin is specific for the substrate casein, and although proteolytic activity is evident in the AGE samples, 50 and 100 % (v/v), the results are not a true indication of

the total protease content within AGE. There are many other proteases that may be present and this particular assay is not specific for a range of proteases, therefore, SDS-PAGE analysis was utilised to observe total proteolytic activity.

4.4.1.1. Determination of the protein content within AGE using the Bradford assay

Due to the dark intense colouring of AGE, protein concentration was only determined for the AGE concentration 3.12 % (v/v) and was found to be 0.002 mg/mL, when determined from the BSA standard curve (Appendix IV). Therefore, it is apparent that there are proteins present however the total protein content cannot be determined from this particular assay.

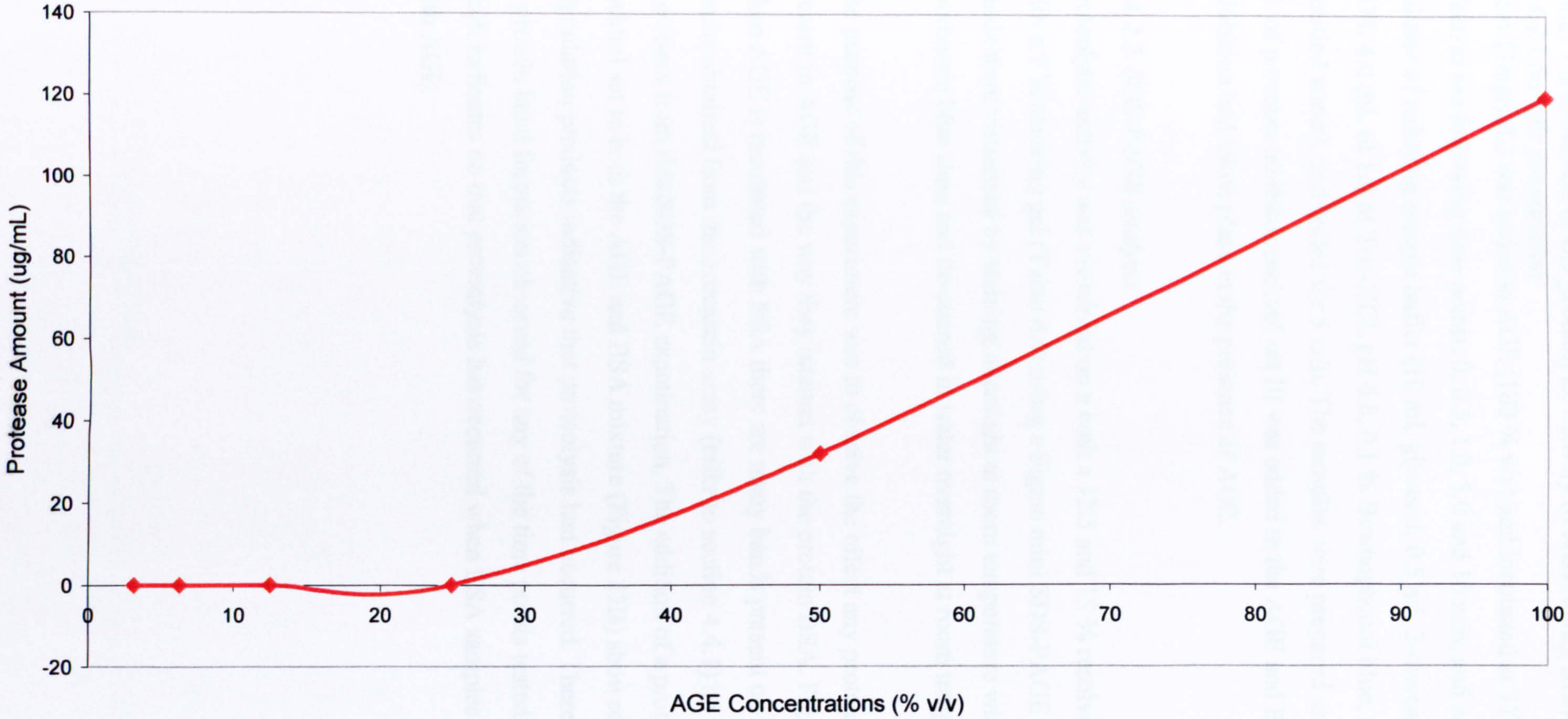

Figure 4.22. Proteolytic activity of AGE, determined by the azocasein assay. AGE concentrations (3.12-100 %, v/v) were incubated at 37°C, for 30 min with azocasein. The reactions were terminated via the addition of TCA. The OD was measured at 450 nm and the activity of AGE was determined from a chymotrypsin standard curve.

4.4.2. Visualisation of the proteolytic activity of AGE using SDS-PAGE

4.4.2.1 Sample preparation

BSA (1mg/mL) was added to AGE (100 % v/v) and incubated at 37°C, aliquots were taken at the following time points: 0, 0.5, 1.0, 5.0 and 10 min, and added to an equal volume of reducing sample buffer (10 mL glycerol, 0.5 mL 2-mercaptoethanol, 1.0 g SDS, 4.0 mL of 1.0 M Tris-HCL pH 6.8, 0.1 % Bromophenol blue, made up with distilled water), and boiled for 5 min. The samples were prepared as above, this time 50 µL of protease inhibitor cocktail set III was added to the AGE and BSA to see if any inhibition had taken place in the presence of AGE.

4.4.2.2. SDS-PAGE analysis

Proteolytic activity was visualised on a both a 12.5 and 15 % resolving gel (Table 4.3) with a 5 % stacking gel (Table 4.4) using a Sigma mini SDS-PAGE gel system. Protein bands were visualised by staining overnight at room temperature with Colodial coomassie blue stain and de-stained in water overnight at room temperature.

The purpose of this experiment was to observe the effect any proteases that may be present in AGE and the way they interact with the protein BSA. Figure 23A shows that when AGE is incubated with BSA there are many bands present on the gel. Unlike the results obtained from the azocasein assay (refer to section 4.4.1) no proteolytic activity is evident from this SDS-PAGE examination. The addition of a protease inhibitor cocktail set to both the AGE and BSA mixture (Figure 23B) showed no apparent degradation products indicative that proteolysis had occurred. There was no difference in protein band formation observed for any of the time points tested. Therefore, these result indicates no that proteolysis has occurred when BSA samples were incubated with AGE.

Table 4.3. Resolving Gel

	12.5 % (mL)	15 % (mL)
30 % Acrylamide	6.25	7.5
1 % Bis-acrylamide	1.56	1.3
1.5 M Tris-HCL pH 8.8	3.75	3.75
20 % SDS	0.15	0.15
Water	3.19	2.2
10 % APS	0.1	0.1
TEMED	0.01	0.01

Table 4.4. Stacking Gel

	5 % (mL)
30 % Acrylamide	2.5
C1 % Bis-acrylamide	3.9
1.0 M Tris-HCL pH 6.8	3.75
20 % SDS	0.15
Water	4.0
10 % APS	0.1
TEMED	0.02
0.1 % Bromophenol blue	0.6

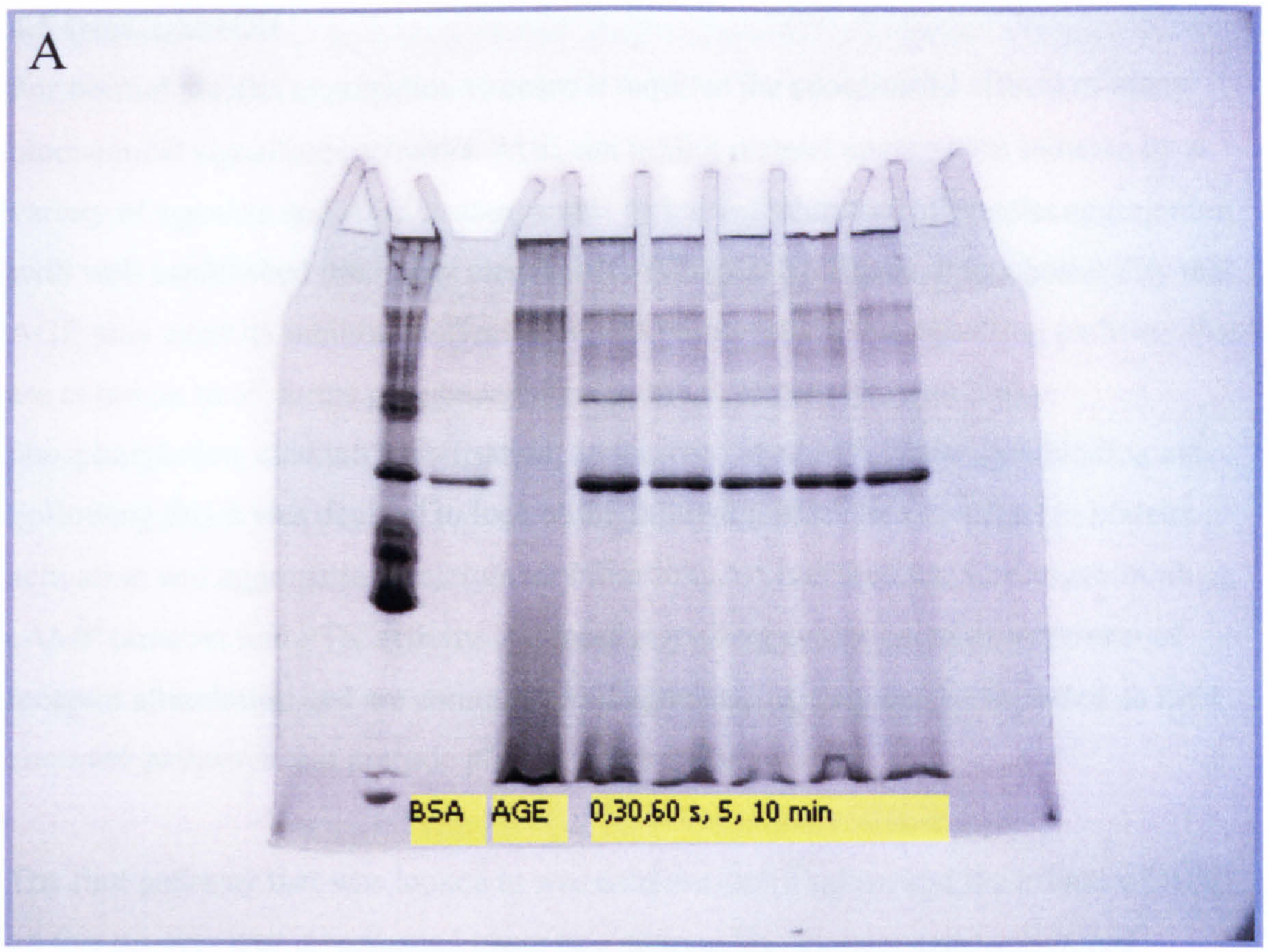

Figure 4.23. The assessment of the proteolytic activity in AGE using SDS-PAGE analysis.
A- the proteolytic activity of AGE, using the protein BSA
B – inclusion of protease inhibitor cocktail set III

4.5 DISCUSSION

For normal platelet aggregation to occur it requires the coordinated effects of many biochemical signalling pathways. AGE can inhibit platelet aggregation induced by a variety of agonists and AGE is comparable to known inhibitors of platelet aggregation with well established inhibitory mechanisms (Chapter 3). Hence, it is a possibility that AGE may exert its inhibitory effect at certain points within the signalling pathway that are common to all forms of agonist stimulation i.e. protein tyrosine kinase phosphorylation, calcium mobilisation, an increase in cAMP, fibrinogen binding etc. Following this it was decided to look at the following processes involved in platelet activation and aggregation: calcium mobilisation; AA metabolism; fibrinogen binding; cAMP turnover and PTK activity. All these signalling events occur downstream of receptor stimulation and are common to all agonists, i.e. they can be regarded as final common pathways that precede platelet aggregation.

The first pathway that was looked at was calcium mobilisation and the effects of AGE on this process was investigated using two approaches: platelet aggregation and fluorescence spectrophotometry. Calcium mobilisation was chosen because earlier studies have looked at the inhibitory effects of garlic constituents and other natural compounds on platelet aggregation (Apitz-Castro *et al.* 1983; Moon *et al.* 2000; Park *et al.* 2004; Qi *et al.* 2000; Rendu *et al.* 1986; Rendu *et al.* 1992; Sheu *et al.* 2003). It was found in this study, that AGE significantly suppressed calcium mobilisation induced by the agonists ADP and A23187. However, significant variation was noted between individuals in their response to individual agonists and this was reflected in the results obtained (Figure 4.6 – 4.7). Thus, the data presented here suggests that AGE may inhibit platelet aggregation by suppressing the mobilisation of calcium ions within platelets. As mentioned previously, Dillon *et al.* (2003) found that AGE has metal chelating properties therefore AGE may act as a chelator of calcium ions during the aggregation process. Another possibility for the observed inhibitory effect is that AGE may alter intracellular signalling events that precede calcium mobilisation, i.e. increase in cAMP activity, fibrinogen receptor expression and binding (Rosado *et al.* 2001) and possibly PTK activity. Stafford *et al.* (2001) reported that aggregation initiated with the calcium ionophore A23187 results in aggregation due to the synergism between the PKC and Ca^{2+} signalling pathways in platelets. However, as reported in chapter 3 no inhibition of platelet aggregation was observed with the inhibitor bisindolymalemide

(PKC inhibitor for all isoforms) on ADP-induced platelet aggregation. In contrast, in the aggregation studies it was found that AGE does inhibit platelet aggregation initiated with the ionophore A23187; this validates the suggestion that AGE is having an inhibitory effect via calcium signalling within the platelet.

This study, although a preliminary one suggests that AGE has an inhibitory effect upon platelet aggregation via the suppression of calcium mobilisation. This occurrence was further validated through more experimentation which showed that calcium ion concentration was significantly inhibited in the presence of AGE (Figure 4.8). Also a reduction in the fluorescence intensity of the calcium ionophore Fura-2/AM in the presence of AGE was exhibited using fluorescence microscopy (Figure 4.20).

The next pathway that was looked at was AA metabolism. Agonists on binding to their membrane receptors cause the hydrolysation of AA from phospholipids to form TXA_2 via COX and TXA_2 synthase. TXA_2 in turn promotes recruitment and further activation of platelets. A literature search conducted showed that most scientists researching natural compounds including garlic found that AA metabolism is an important pathway with regards to platelet aggregation. The underlying mechanism proposed for inhibition of platelet aggregation via the AA metabolic pathway is that it halts the activity of COX (Ali 1995; Ali *et al.* 2000; Apitz-Castro *et al.* 1983; Bordia *et al.* 1978; Chan *et al.* 2002; Jin *et al.* 2005; Kleijnen *et al.* 1989; Koo *et al.* 2001; Macdonald and Langer 2000; Makheja *et al.* 1980; Moon *et al.* 2000; Qi *et al.* 2000; Shah *et al.* 1999; Sheu *et al.* 2003; Son *et al.* 2004; Srivastava 1984; Srivastava 1986; Srivastava and Tiwari 1980; Srivastava and Tyagi 1993; Stampfer *et al.* 1987; Vanderhoek *et al.* 1980).

In this study AA metabolism was first monitored using the method outlined by Jin *et al.* (2002). In their study platelets were loaded with [^{3}H]-AA, washed and stimulated with an agonist. Platelet aggregation was stopped via a stopping solution, centrifuged and the supernatant monitored for radioactivity. It was found initially that AGE had a 'quenching' effect upon the radiolabel ^{14}C-AA during scintillation counting. This resulted in this method being discarded for the more classical approach of TLC. Numerous problems were encountered when trying to measure the effects of AGE on AA metabolism and these included the loss of radiolabel during washing steps and extraction with ether.

It was often difficult to identify radiolabelled metabolites from AA metabolism via liquid scintillation counting from the process of TLC. A possible explanation for this occurrence is that AA is a platelet agonist; addition of AA could inadvertently activate platelets. Thus, addition of ADP to the platelet suspensions resulted in the amplification of the initial platelet response to AA (platelets may have already gone through the aggregation process). It was found that AGE did not have a significant effect upon AA metabolism during platelet activation initiated with ADP. These results were compared to the agonist thrombin and again no inhibitory effect was found. These results suggest that ADP-induced platelet aggregation can occur independently of AA metabolism. The aggregation studies reported in chapter 3 using the classical inhibitors: aspirin and indomethacin also showed that no inhibition occurred during ADP-induced platelet aggregation. Both of these inhibitors inhibit AA metabolism by suppressing the actions of COX. Thus, a TXA_2 independent pathway must contribute to platelet aggregation induced by ADP. It is possible that AGE may have an inhibitory effect upon AA metabolism as other studies have shown that other garlic preparations and its constituents and other natural compounds do inhibit this pathway. However, contradictory results have been obtained in this study for ADP-induced platelet aggregation. Mangin *et al.* (2004) showed that the ADP receptor is important in platelet activation during collagen-induced platelet aggregation when TXA_2 formation is prevented. Thus, platelet aggregation can still occur even when a major signalling pathway has been suppressed.

It is well known that PGE_1 is an inhibitor of platelet aggregation and is used clinically in the treatment of atherosclerosis (Fisch *et al.* 1995; Katzenschlager *et al.* 1992; Kinlough-Rathbone *et al.* 1970; Kikura *et al.* 2000; Koga *et al.* 2002). It has also been found that when PGE_1 is added to aggregated platelets that the process is reversed (Kikura *et al.* 2000) and the platelets disaggregate. AGE was tested to see if like PGE_1 it could cause the disaggregation of ADP activated platelets. The reasoning behind this experiment is that platelet activity is regulated in part by cyclic adenosine monophosphate (cAMP), and an increase in this substance reduces platelet activity by inhibiting various morphological and chemical changes within the cell (Kikura *et al.* 2000; Kinlough-Rathbone *et al.* 1970). In the body PGE_1 working synergistically with the endothelial cells stimulates AC to produce more cAMP. Thus, is AGE working in a

similar manner to that of PGE_1, in that does AGE result in an increase in cAMP levels which results in inhibition of platelet aggregation?

The effect of AGE on platelet adhesiveness to immobilised fibrinogen was investigated. GPIIb/IIIa plays a major role in the regulation of platelet aggregation during haemostasis. Upon activation by an agonist such as ADP a signalling process is initiated known as "inside-out" signalling, which causes a conformational change within the receptor and this increases the affinity of GPIIa/IIIb for its ligand fibrinogen. Upon fibrinogen binding the receptor undergoes further shape changes and through another process termed "outside-in" signalling GPIIa/IIIb results in the amplification of platelet aggregation (Fullard 2004; Naimushin and Mazurov 2003; Peerschke and Zucker 1981; Shattil *et al.* 1998).

Maayani *et al.*, (2001) reported that disaggregation of ADP-induced platelet aggregation is associated with the dissociation of fibrinogen from its receptor on the platelet surface. It has also been found that the organosulphur compound ajoene, a constituent of garlic inhibits fibrinogen binding and subsequently platelet adhesion (Tapiero *et al.* 2004). The suggested mechanism for this antagonism is a sulfhydryl group-mediated effect as the GPIIb-IIIa receptor has a high content of –SH groups. This is due to organosulphur compounds being readily cleared *in vivo*, separating into –SH compounds as reported by Steiner and Lin (1998). Hence, this occurrence could possibly explain the reduction in platelet adhesion to fibrinogen. It was found in this study that AGE did reduce the amount of platelets bound to immobilised fibrinogen compared to that of the controls (Figures 4.14-4.16). It has been reported by Tapiero *et al.* (2004) that un-stimulated platelets express the GPIIb-IIIa complex at their surface, and that this complex is unable to bind fibrinogen until the platelets have been activated. Activation causes a change within the receptor that allows for fibrinogen to be bound and cross-linked with other platelets resulting in the formation of an aggregate (Shattil *et al.* 1998). This is why the platelets in this study were activated with ADP in the presence of fibrinogen. Bovine fibrinogen was used instead of human fibrinogen because they both contain the same RGD sequences, therefore human platelets when activated are able to adhere to Bovine fibrinogen and form aggregates with other platelets.

similar manner to that of PGE_1, in that does AGE result in an increase in cAMP levels which results in inhibition of platelet aggregation?

The effect of AGE on platelet adhesiveness to immobilised fibrinogen was investigated. GPIIb/IIIa plays a major role in the regulation of platelet aggregation during haemostasis. Upon activation by an agonist such as ADP a signalling process is initiated known as "inside-out" signalling, which causes a conformational change within the receptor and this increases the affinity of GPIIa/IIIb for its ligand fibrinogen. Upon fibrinogen binding the receptor undergoes further shape changes and through another process termed "outside-in" signalling GPIIa/IIIb results in the amplification of platelet aggregation (Fullard 2004; Naimushin and Mazurov 2003; Peerschke and Zucker 1981; Shattil *et al.* 1998).

Maayani *et al.*, (2001) reported that disaggregation of ADP-induced platelet aggregation is associated with the dissociation of fibrinogen from its receptor on the platelet surface. It has also been found that the organosulphur compound ajoene, a constituent of garlic inhibits fibrinogen binding and subsequently platelet adhesion (Tapiero *et al.* 2004). The suggested mechanism for this antagonism is a sulfhydryl group-mediated effect as the GPIIb-IIIa receptor has a high content of –SH groups. This is due to organosulphur compounds being readily cleared *in vivo*, separating into –SH compounds as reported by Steiner and Lin (1998). Hence, this occurrence could possibly explain the reduction in platelet adhesion to fibrinogen. It was found in this study that AGE did reduce the amount of platelets bound to immobilised fibrinogen compared to that of the controls (Figures 4.14-4.16). It has been reported by Tapiero *et al.* (2004) that un-stimulated platelets express the GPIIb-IIIa complex at their surface, and that this complex is unable to bind fibrinogen until the platelets have been activated. Activation causes a change within the receptor that allows for fibrinogen to be bound and cross-linked with other platelets resulting in the formation of an aggregate (Shattil *et al.* 1998). This is why the platelets in this study were activated with ADP in the presence of fibrinogen. Bovine fibrinogen was used instead of human fibrinogen because they both contain the same RGD sequences, therefore human platelets when activated are able to adhere to Bovine fibrinogen and form aggregates with other platelets.

The principle behind this study was that Rose Bengal, a biological stain and chromium-51 are taken up by platelets that are bound to the fibrinogen matrix upon the well surface. Upon lysis of the platelets the Rose Bengal and chromium-51 are released and the absorbance is measured spectrophotometrically and radioactivity via liquid scintillation counting, respectively. The amount of absorbance/radioactivity from the platelets treated with AGE is compared to that of the controls. A reduction in the optical density and radioactive count readings indicates that there is a reduction in the amount of platelets that are bound to the matrix and thus AGE has prevented adhesion and subsequent formation of a platelet aggregate. Although the method is a simple one it shows that AGE does have an effect upon platelet adhesion and the formation of aggregates.

The direct effect of AGE upon fibrinogen was also examined and it was found that AGE at all the concentrations used significantly inhibited platelet adhesion when activated with ADP. Therefore, AGE affects the binding properties of fibrinogen in its ability to form a platelet aggregate. To investigate this further flow cytometry was used to monitor the effect of AGE on fibrinogen binding using the antibody PAC-1 which is specific for the receptor GPIIb/IIIa when it undergoes its conformational change and binds fibrinogen through agonist stimulation. Platelets activated with ADP have a higher incidence of PAC-1 binding compared to those that are inactivated (Figure 4.18). In the presence of AGE the binding of PAC-1 to the activated form of GPIIb/IIIa is significantly reduced when compared to those that had been activated with ADP. Thus, AGE is having an inhibitory effect on platelet aggregation via fibrinogen binding.

As mentioned previously it was assumed that AGE may be acting in a similar manner to PGE_1, as PGE_1 when added to pre-aggregated platelets results in disaggregation. It is known that the main inhibitory mechanism of PGE_1 is through an increase in the levels of cAMP within the platelet which in turn inhibits the release of calcium ions from intracellular stores and increases calcium ion sequestration back into the dense tubular system. Also, the affinity of the fibrinogen receptor can be prevented or reversed by compounds such as PGE_1 and PGI_2 that stimulate an increase in cAMP. Thus the effect of AGE on intracellular cAMP levels was examined. The results obtained show that AGE when added to platelets caused a significant increase ($P<0.01$) in the intracellular levels of cAMP in both basal and activated platelets. Figure 4.9A shows that basal

levels are high in the AGE treated samples and as expected in the PGE_1 treated groups also. The increased levels of cAMP are still observed upon agonist stimulation (Figure 4.9B) indicating that inhibition of platelet aggregation may be in part due to an increase in cAMP; leading to a reduction in intracellular calcium concentration and the prevention of fibrinogen binding to its receptor. A simple aggregation study was carried out to ensure that the actions of AGE were directly upon cAMP and not cAMP-dependent PDEs. IBMX, a PDE inhibitor when added to AGE resulted in a significant increase in the inhibition of platelet aggregation compared to AGE alone

Activators of cAMP can decrease the levels of calcium within the aggregating platelet, and calcium in known to be important in many intracellular signalling processes, such as the fibrinogen receptor undergoing its conformational change. Therefore it is possible that the preincubation of platelets with AGE results in inhibition brought about by an increase in cAMP, which in turn reduces calcium levels, ultimately leading to the fibrinogen receptor not being able to undergo its conformational change. It was reported in a study conducted by Samal and Loiko (2000), that N-ethylmaleimide caused the disaggregation of both ADP- and thrombin-induced aggregated platelets. In their study they found that the disaggregation observed, was the result of a system that removed calcium from activated platelets in the cytosol. Both preliminary and secondary studies have shown that platelet incubation with AGE leads to a suppression in calcium mobilisation upon the initiation of platelet aggregation via the agonist ADP. This result further supports the findings presented here suggesting that AGE inhibits ADP-induced platelet aggregation via cAMP pathway. Many other researchers looking at different dietary extracts such as ginger, gingko biloba etc. have also found that platelet aggregation in inhibited via an increase in the intraplatelet levels of cAMP (de Lange *et al.* 2003; Duttaroy *et al.* 2004; Innes *et al.* 2003; Moon *et al.* 2000; Osmont *et al.* 2003; Shah *et al.* 1999).

Proteolytic activity experiments utilising the azocasein assay and SDS-PAGE analysis were conducted with AGE. The main purpose of this investigation was to examine another potential mechanism by which AGE may exert its inhibitory effect with regards to platelet aggregation. It may be possible that direct contact of the platelet with AGE may directly act on a specific receptor important in platelet aggregation. The potential proteolytic activity of AGE could be via the cleavage of receptor proteins, e.g. at the

fibrinogen receptor GPIIb/IIIa to prevent fibrinogen binding and subsequent platelet aggregate formation. The experiments show that AGE does exhibit signs of proteolysis through its interactions with the protein casein (azocasein assay, Figure 4.22) but not via incubation with BSA (SDS-PAGE analysis, Figure 4.23).

In conclusion this study has shown through *in vitro* testing that AGE inhibits fibrinogen binding via the receptor GPIIb/IIIa, suppresses the mobilisation of calcium within the platelet, increases the intraplatelet levels of cAMP and it also displays proteolytic properties through its interactions with casein.

AGE has exhibited a wide range of potential inhibitory mechanisms with regards to platelet aggregation (Figure 4.24). This may be due to the complex make-up of the AGE preparation, in that the components contained within it act synergistically on different signalling pathways involved in the activation and aggregation process to bring about the observed inhibitory effect on ADP-induced platelet aggregation. This needs to be investigated further in a clinical trial.

4.6. SUMMARY OF RESULTS

- Platelet aggregation initiated with the agonists ADP and A23187. This was noted through fluorescence spectrophotometry and fluorescence microscopy.
- AGE had no effect upon AA-uptake and metabolism during ADP-induced platelet aggregation.
- AGE inhibited fibrinogen binding to its receptor GPIIb/IIIa during platelet aggregation induced with ADP. This was validated with the use of four independent methods: disaggregation, a simple adhesion assay, flow cytometry and SEM imaging.
- AGE significantly increased intraplatelet levels of cAMP when compared to the inhibitor PGE_1.
- AGE potentially showed signs of proteolytic activity as determined by the azocasein assay.

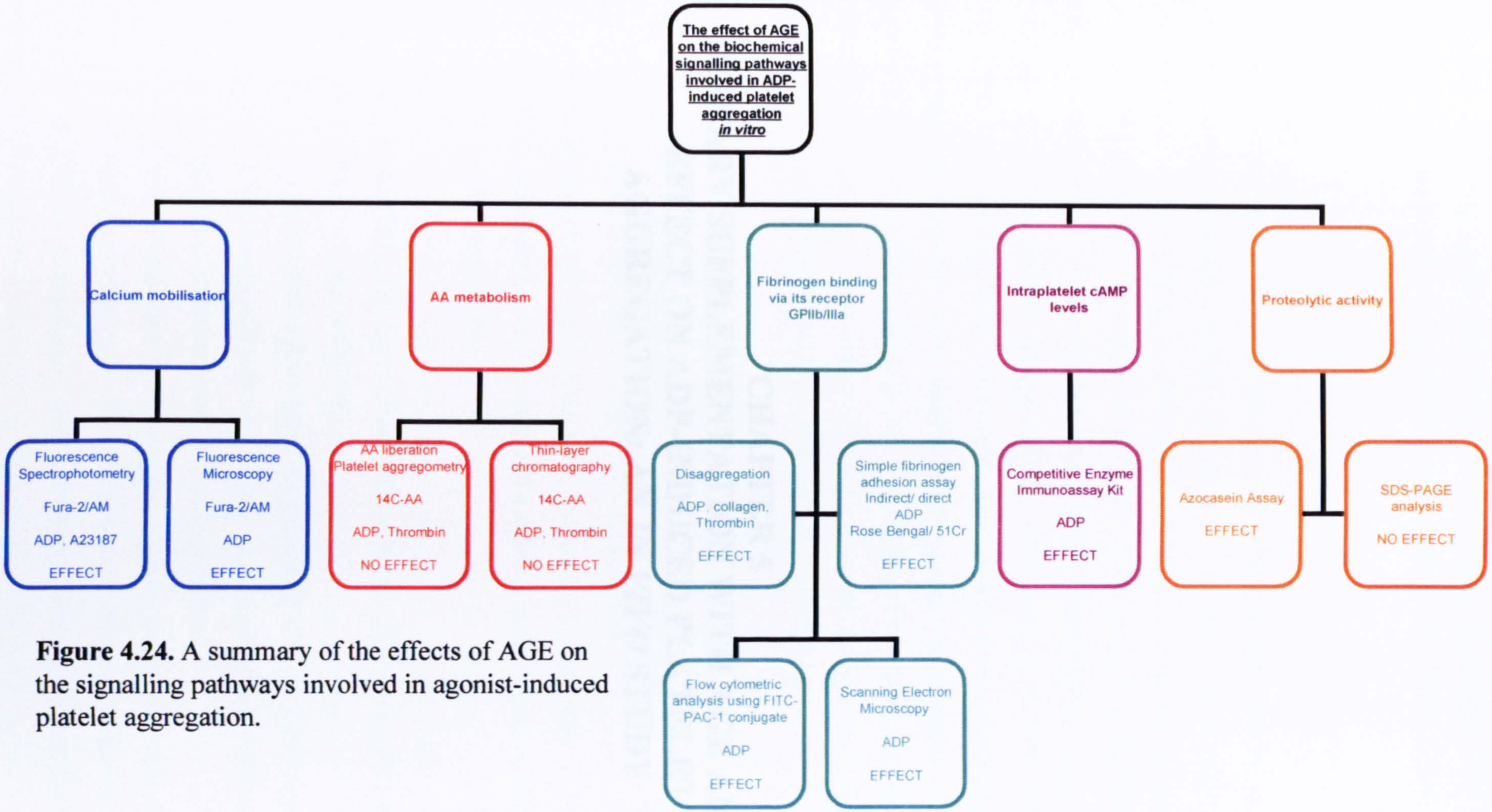

Figure 4.24. A summary of the effects of AGE on the signalling pathways involved in agonist-induced platelet aggregation.

CHAPTER 5

DIETARY SUPPLEMENTATION WITH AGE AND ITS EFFECT ON ADP-INDUCED PLATELET AGGREGATION: AN *IN VIVO* STUDY

5. DIETARY SUPPLEMENTATION WITH AGE AND ITS EFFECT ON AGONIST-INDUCED PLATELET AGGREGATION: AN *IN VIVO* STUDY

5.1. INTRODUCTION

Garlic, especially the dietary supplement AGE has been shown to inhibit agonist-induced platelet aggregation both *in vitro* and *in vivo* by numerous scientific studies over the past 30 years (Table 1.2, Chapter 1, page 16-17). *In vivo* studies using AGE have found that ingestion of AGE over a set time period results in a significant marked decrease in the ability of human platelets to aggregate in response to various agonists (Rahman and Billington 2000; Steiner and Lin 1998; Steiner *et al.*, 1996; Steiner and Lin 1994; Steiner and Li 2001). The present study has also shown that AGE inhibits platelet aggregation *in vitro* when platelets are stimulated with a variety of aggregating agents (Chapter 3, section 3.2.1, page 55). The findings from these aggregation assays (Chapter 3) suggested that the observed inhibition was the result of AGE acting on signal transduction pathways common to all forms of agonist stimulation.

In chapter 4, the effect of AGE on the following biochemical pathways were reported:-

- Calcium mobilisation (Chapter 4, section 4.2.1, page 98)
- AA metabolism (Chapter 4, section 4.4.2, page 107)
- Intracellular cAMP levels (Chapter 4, section 4.4.3, page 114)
- Fibrinogen binding – the formation of a stable platelet aggregate (Chapter 4, section 4.4.4, page 118)

The only pathways that AGE had an observed inhibitory effect upon ADP-induced platelet aggregation was on the following:

- calcium moblisation – platelets pretreated with AGE had significantly lower levels of calcium ions compared to the controls (untreated platelets) when measured with a fluorimeter.
- Intracellular cAMP – incubation of platelets with AGE resulted in an increase in the levels of cAMP following agonist stimulation.
- Fibrinogen binding – AGE reduced the formation of stable platelet aggregates.

All the above experiments were conducted *in vitro* and were the result of AGE being directly incubated with platelets contained in PRP (platelet-rich plasma). Hence, the question arises would AGE have the same effect in an *in vivo* situation?, as there are many other biological and chemical processes, which occur in the body that may effect the inhibitory actions of AGE upon platelet function.

This present study is novel and differs greatly from preceeding clinical trials conducted on AGE (Rahman and Billington 2000; Steiner and Lin 1998; Steiner *et al.,* 1996; Steiner and Lin 1994; Steiner and Li 2001) in that the major aim here is to establish at which point(s) in the signal transduction pathways does AGE exerts its inhibitory effect to reduce agonist-induced platelet aggregation. To investigate this further and to ascertain whether AGE does have a definite inhibitory effect upon these pathways (intracellular cAMP and fibrinogen binding) a clinical trial was conducted.

5.2. OVERVIEW OF STUDY

In this study the effect of AGE on platelet aggregation both *ex vivo* and *in vivo* was investigated this was done by the following:

- Whole human blood was directly incubated with AGE. Platelets were removed via centrifugation, and platelet aggregation was initiated with the aggregating agent ADP *ex vivo*.

As mentioned in previously (Chapter 3) AGE was directly incubated with platelets contained in platelet-rich plasma. This direct incubation resulted in the significant inhibition of platelet aggregation initiated by a variety of agonists. The purpose of this particular study is to investigate whether incubation of whole blood with AGE will result in inhibition of platelet aggregation *ex vivo*.

- Clinical trial – dietary supplementation with AGE over a set time period.

For the clinical trial volunteers who matched the selection criteria were chosen from the university population and blood samples were taken at Day 0. The volunteers were

asked to ingest 5 mL/ day of AGE (equivalent to 1200 mg of the powder form of AGE and, is the recommend daily amount that has been reported not to cause any serious side-effects and has also been deemed safe following toxicological studies carried out by Wakunaga of America – www.kyolic.com) everyday for 14 days. After AGE consumption (Day 14), blood samples were taken, and again following a washout period of 14 days (Day 28). Blood samples were taken at Days 0, 14 and 28 and were tested for the parameters listed below. During the clinical trial (28 days) subjects were asked to continue following their usual diet and lifestyle, including: cigarette smoking, alcohol intake and dietary supplementation.

1. Platelet aggregation initiated with the agonists: ADP, AA, adrenaline and collagen (for methods see Chapter 2, section 2.4.1, page 31). *In vitro* studies (Chapter 3) showed that AGE inhibited platelet aggregation in a dose-dependent manner for the agonists listed above. Another reason for the choice of agonists is that in other studies (*in vitro/ in vivo*) examining the effects of garlic upon platelet aggregation these have been widely used (Rahman and Billington 2000; Steiner and Lin 1998; Steiner *et al.,* 1996; Steiner and Lin 1994; Steiner and Li 2001).
2. GPIIb/IIIa receptor binding using flow cytometry (for detailed methods see Chapter 4, section 4.2.3.4, page 129). Fibrinogen binding was chosen because during *in vitro* (Chapter 4) testing AGE significantly reduced the formation of stable platelet aggregates when monitored using flow cytometry. In addition, sample preparation is relatively easy for this particular assay and it can be done in whole blood.
3. cAMP levels (for methods see Chapter 2, section 2.4.4, page 44) The *in vitro* study showed that AGE significantly increased intraplatelet levels of cAMP.
4. Total cholesterol (for methods refer to section, page 169) was monitored in this trial to see if there was any reduction in levels following AGE treatment. AGE is reported to exert hypolipidemic properties (Liu and Yeh, 1999; Yeh and Liu, 2001).

The above assays were chosen because they do not require copious amounts of platelets, are relatively simple, do not require long incubation periods, and are easy to conduct

and to investigate if AGE has any clinical relevance. During *in vitro* testing (Chapter 4) it was found that AGE significantly reduced fibrinogen binding and increased cAMP levels, thus it was also decided to monitor these particular assays during the clinical trial.

It is important to note that platelet function can be affected by a variety of parameters from medication (i.e. aspirin, NSAIDs, and other anti-platelet drugs), diet, exercise, excessive alcohol, gender (females – menstruation), age, dietary supplements and smoking. Therefore, for the purpose of this study volunteers were asked to refrain from taking any medication that interfered with platelet function for 2 weeks prior to blood donation. The only variable that was under investigation was the effect of AGE upon platelet aggregation following AGE administration for a set time period. All volunteers were apparently healthy with no pre-existing medical conditions that affect platelet function and were within the age range 18-65 years, as this is reflective of the ages present within the university population.

5.3. RESULTS

5.3.1. The *ex vivo* effect of AGE on platelet aggregation induced with ADP

AGE inhibits platelet aggregation *in vitro* when PRP is incubated with AGE for 10 min at 37°C, and stimulated with a variety of agonists (Chapter 3, section 3.2.1, page 55). In whole blood there are many other cells, proteins and chemical substances that could possibily interact with AGE and affect its inhibitory actions on platelet aggregation. Therefore, it is important to establish that pre-treatment of whole blood with AGE results in the inhibition of agonist-induced platelet aggregation using platelet aggregometry.

In this experiment, AGE at various concentrations was incubated directly with whole blood for 15 min at 37°C. To the control an equal volume of PBS was added to the whole blood and treated in the same manner as the AGE samples. The platelet-rich plasma was removed by centrifugation from the whole blood and platelet aggregation was initiated via the addition of the agonist ADP. Platelet aggregation was monitored using platelet aggregometry in a PAP-4D Platelet Aggregation Profiler.

The results (Figure 5.1) are interesting in that they indicate that ADP-induced platelet aggregation is dose-dependently reduced when whole blood is directly incubated with AGE at all the concentrations tested (12.5-50 %, v/v), compared to the PBS control sample. Statistical significance ($P<0.05$) was noted for AGE concentrations at 25 and 50 % (v/v). The results follow a pattern in that the higher the AGE concentration the more inhibition is observed.

The results presented in this study show that a much higher dose of AGE is required for inhibition of platelet aggregation in whole blood in comparison to the levels monitored during *in vitro* testing (25 %, v/v – maximum dose) in PRP (Chapter 3). It would not be practical or feasible to use this kind of dosing regime during a clinical trial, as subjects would have to ingest approximately 0.5 litres of AGE/ day. Therefore, for the purpose of the clinical trial subjects will be following the dosage recommended by the manufacturer of 5mL/day as this dose has been shown to be effective in eliciting a response in other clinical studies using AGE (Rahman and Billington 2000; Steiner and Lin 1998; Steiner *et al.*, 1996; Steiner and Lin 1994; Steiner and Li 2001).

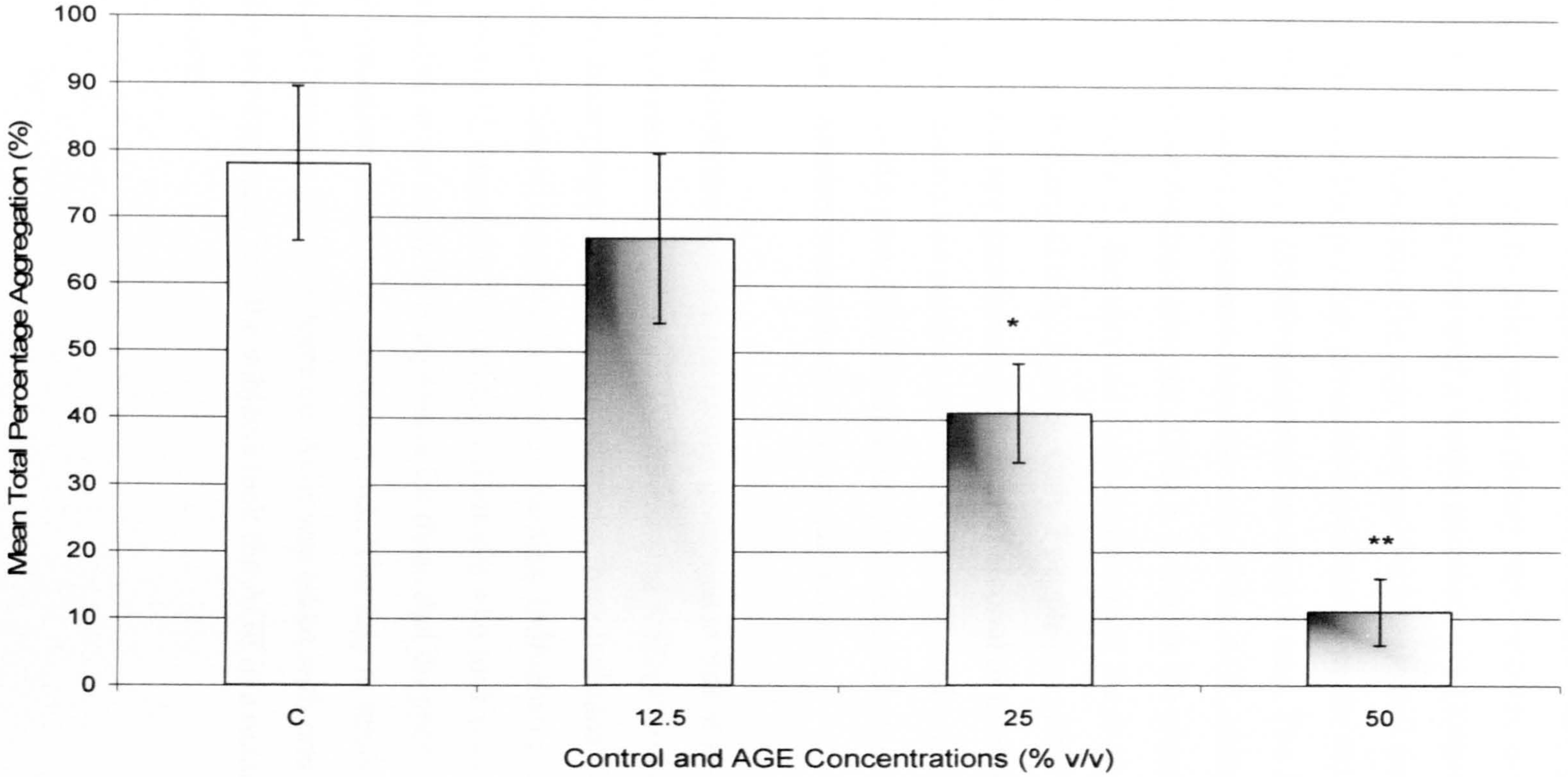

Figure 5.1. The *ex vivo* effect of AGE on ADP-induced platelet aggregation. Whole human blood was incubated with AGE (12.5-50 %, v/v) for 15 min at 37°C. PRP was removed from the whole blood via centrifugation. Platelet aggregation was initiated with ADP (8 µmol/L), and the reaction was allowed to run for 5 min. Aggregation curves were analysed for total percentage aggregation and values are expressed as means ± SEM, n=4. Statistically significant *P<0.05, **P<0.01, when compared to the PBS control sample.

5.3.2. Clinical trial – dietary supplementation with aged garlic extract

20 volunteers were recruited randomly from the university population at John Moores and were recruited for this clinical dietary supplementation trial. Before the start of the trial all participants answered a simple questionnaire (Appendix VII) regarding their physical characteristics (i.e. age, average weekly alcohol consumption, number of cigarettes smoked per day, prescribed medication intake etc). From the original 20 volunteers only 17 (85 %) completed the clinical trial. The 3 volunteers that withdrew from the study complained about the taste of the AGE, claiming that it was too strong to ingest without feeling nauseous. Table 5.1, depicts the physical characteristics generated from this study. 11 females and 6 males undertook the study with the mean age of the participants being 41 ± 3.25 years. Only 3 people out of the 17 were smokers and 14 drank on average approximately 6 units of alcohol or more per week during the trial. 3 of the volunteers had medical conditions that required prescribed medication (the illnesses and the prescribed medication were revealed on completion of the trial when the questionnaires were returned for examination).

Data analysis showed that the results generated in this study were not affected by those taking prescribed medications for pre-existing medical conditions and, 6 regularly took other dietary supplements on a daily basis, these included Cod Liver Oil capsules, omega-3 fish oil capsules and multi-vitamins. Individual data generated from this study is shown in Appendix VIII all the volunteers who took prescribed medications were included in the study to compensate for those that dropped out. The AGE given to all the volunteers came from the same batch. The way in which AGE was administered varied between the volunteers i.e. AGE was taken with orange juice/ tomato juice, neat, after evening meal, and the subjects took the AGE in a manner that was easy for them to tolerate.

Table 5.1. Physical characteristic data of participants in the clinical trial

Physical Characteristic	Number
Total number of volunteers	17
N° of females	11
N° of males	6
Mean AGE of volunteers	41 ± 3.2 years
N° of smokers	3
N° of volunteers who drank more than 6 units of alcohol/ week	14
N° of volunteers taking prescribed medication for an illness that does not affect platelet function	3
N° of volunteers who take other dietary supplements on a daily basis	6

5.3.2.1. The effect of AGE on agonist-induced platelet aggregation in vivo

Dietary supplementation with AGE significantly ($P<0.05$) reduced platelet aggregation initiated with the agonists: ADP, adrenaline and collagen (Figure 5.2). The results indicate that for each of the agonists: ADP (76 % ± 3.7), adrenaline (59 % ± 5.6) and collagen (77 % ± 3.5) maximal platelet aggregation (>60 %) is observed at Day 0. Following AGE administration for 14 days (5 mL/day), platelet aggregation is significantly reduced upon agonist stimulation with the following aggregating agents: ADP (63 % ± 4.7), adrenaline (30 % ± 6.9) and collagen (58 % ± 7.1). On Day 28, following the two week washout-out period, the levels of platelet aggregation observed for each of the agonists was comparable to those obtained at Day 0: ADP (79 % ± 3.6), adrenaline (60 % ±6.9) and collagen (76 % ± 5.1). These results suggest that AGE administration of 5 mL/day for 14 days reduces platelet aggregation and following a suitable washout-period (2 weeks) the amount of aggregation monitored returned to a level similar to that seen at the baseline (Day 0) for the aggregating agents ADP, adrenaline and collagen. However, the agonist AA failed to initiate maximal aggregation in the group studied. There was no significant inhibition of platelet aggregation following AGE administration with this particular agonist. Although from Figure 5.2 it is apparent that following AGE consumption there is a reduction in platelet aggregation initiated with AA it was deemed not significant ($P>0.05$), as the agonist failed to produce maximal aggregation.

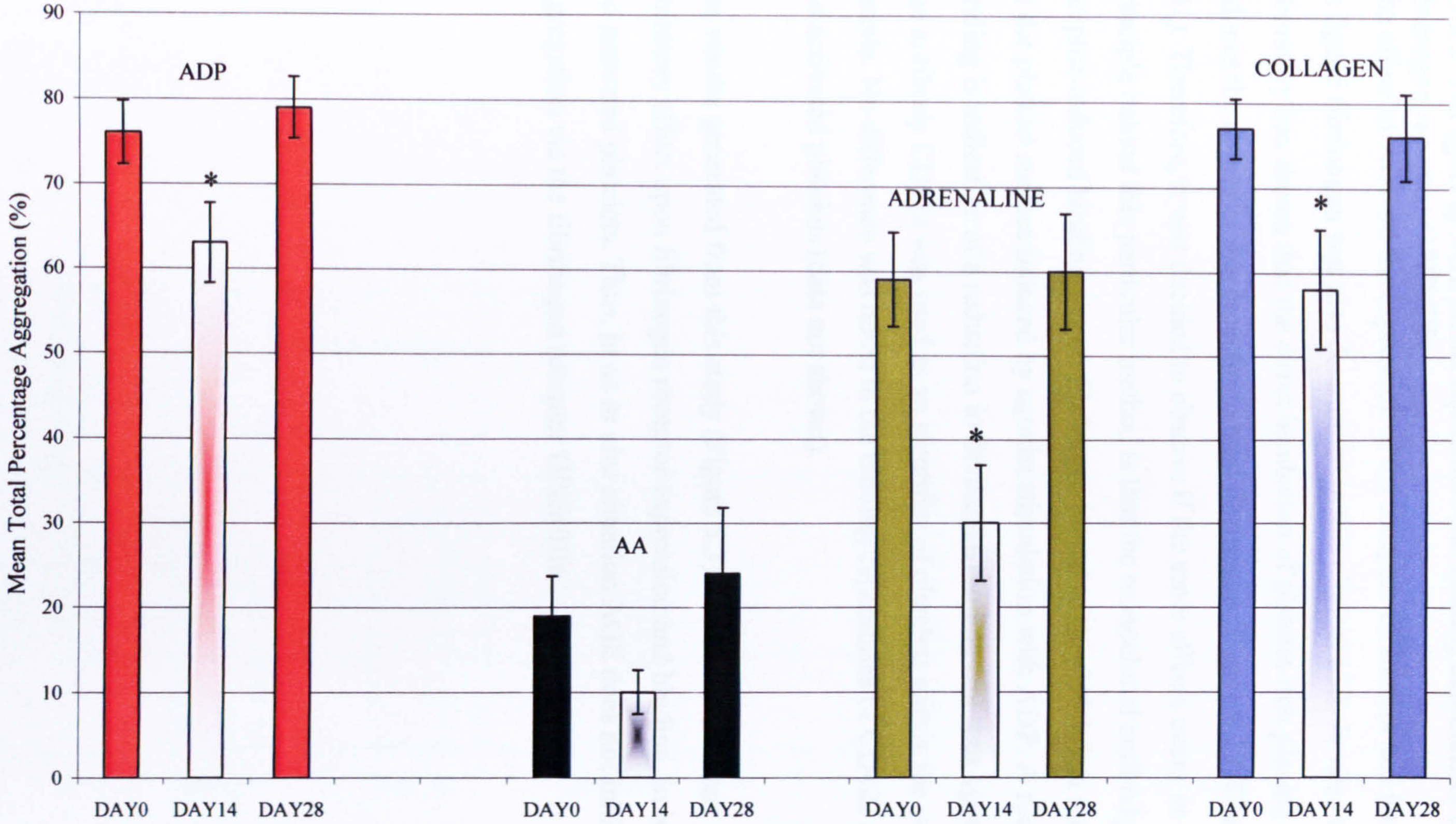

Figure 5.2. Agonist-induced platelet aggregation in humans before (Day0), after AGE administration (Day14), 5mL/day for 14 days and, after a washout period of 14 days (Day28). Platelet aggregation was initiated by the addition of the following agonists: ADP (8 µmol/L), AA (250 µg/mL), adrenaline (20 µmol/L) and collagen (190 µg/mL) to platelet-rich plasma. Aggregation curves were analysed in duplicate for total percentage aggregation (%). Values are presented as means ± SEM, n=17 (Day0, Day14), n=16 (Day28). *Significantly different ($P < 0.05$) when compared to before (Day 0) and after AGE ingestion (Day 28). AA showed no response when compared to ADP, adrenaline and collagen. There was no statistical significance noted between Day 0 and Day 28 values ($P > 0.05$).

5.3.2.2. The effect of AGE on the expression and binding capabilities of the fibrinogen receptor GPIIb/IIIa

The effect of AGE on the expression of the receptor GPIIb/IIIa and its ability to bind to its ligand fibrinogen was also determined in this clinical trial. *In vitro* testing in our laboratory has shown that the direct incubation of platelet-rich plasma with AGE reduces the ability of this receptor to bind fibrinogen (Figure 4.19, Chapter 4, page 131). Therefore, it was decided to observe if the same effects occur *in vivo*. The principle behind this particular method is that the monoclonal antibody PAC-1 detects a receptor-induced binding site on fibrinogen bound to the GPIIb/IIIa receptor complex on the platelet surface induced by agonist stimulation with ADP. A reduction in PAC-1 binding is indicative of a reduction in fibrinogen binding and thus aggregate formation. The antibody CD42a was used as an identifier of platelets within the whole blood sample. No difference was noted in the binding capabilities of CD42a in both resting and activated platelets (data not shown).

The results generated from this study (Figure 5.3) show that AGE has no significant inhibitory effect upon fibrinogen receptor expression and binding, in both inactivated and activated platelets. Thus, in an *in vivo* situation AGE does not inhibit platelet aggregation via the fibrinogen receptor GPIIb/IIIa..

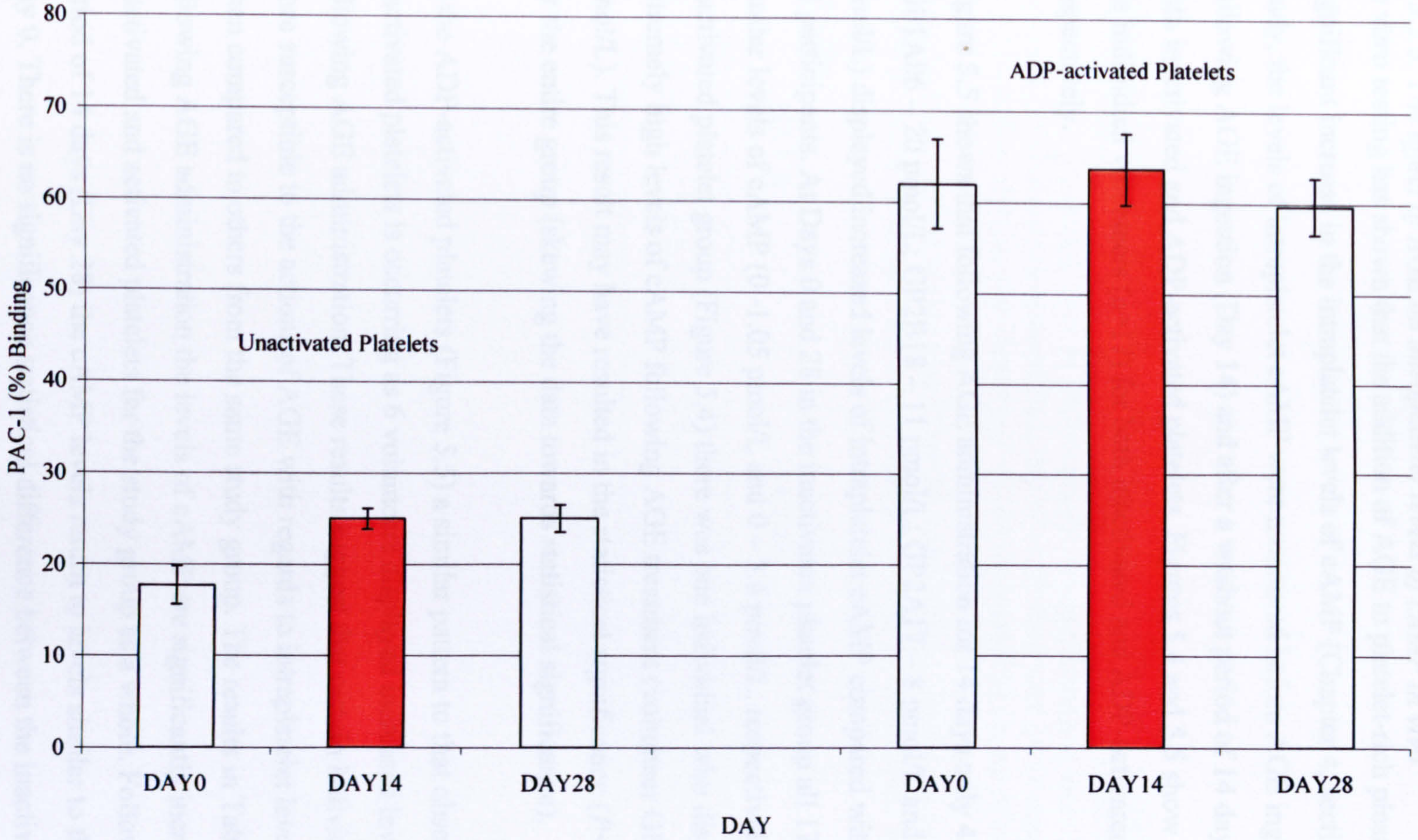

Figure 5.3. The *in vivo* effect of AGE on the expression and binding capabilities of the fibrinogen receptor GPIIb/IIIa in both inactivated and ADP-activated platelets. The binding of fibrinogen to its receptor was monitored using the antibody PAC-1 FITC which is specific for this receptor. Values are expressed as percentages ± SEM, n=17 (Day 0 and Day 14), n=16 (Day 28). No statistical significance ($P > 0.05$) was found between Day 0 and Day 14 (following AGE administration).

5.3.2.3. The effect of AGE on intraplatelet levels of cAMP in vivo

In vitro testing has shown that the addition of AGE to platelet-rich plasma results in a significant increase in the intraplatelet levels of cAMP (Chapter 4, section . In this study, the levels of intraplatelet cAMP were monitored before AGE ingestion (Day 0), following AGE ingestion (Day 14) and after a washout period of 14 days (Day 28), in both inactivated and ADP-activated platelets. Figures 5.4 and 5.5 show the results for the individual volunteers (n=17) for both inactivated and ADP-activated platelets, respectively.

Figure 5.5 shows that following AGE administration for 14 days only 4 volunteers (GP1A06 – 20 pmol/L; GP2B18 – 11 pmol/L; GP2A17 – 8 pmol/L and GP1B224.5 pmol/L) displayed increased levels of intraplatelet cAMP compared with the remaining 13 participants. At Days 0 and 28 in the inactivated platelet group all 17 volunteers had similar levels of cAMP (0 -1.05 pmol/L and 0 – 3.6 pmol/L, respectively). In the inactivated platelet group (Figure 5.4) there was one individual who displayed extremely high levels of cAMP following AGE treatment (volunteer GP1A06 – 20 pmol/L). This result may have resulted in the statistical significance ($P<0.05$) observed for the entire group (skewing the data towards statistical significance).

In the ADP-activated platelets (Figure 5.5) a similar pattern to that observed in the inactivated platelets is occurring as 6 volunteers displayed increased levels of cAMP following AGE administration. These results suggest that certain individuals may be more susceptible to the actions of AGE with regards to intraplatelet levels of cAMP when compared to others from the same study group. The results in Table 5.1 show that following AGE administration the levels of cAMP are significantly increased in both inactivated and activated platelets for the study group as a whole. Following a washout period of 14 days (Day 28) the cAMP levels return to levels similar to those observed at Day 0. There is no significance statistical difference between the inactivated and activated platelet groups for any of the days tested.

Table 5.2. Intracellular cAMP concentration (pmol/L) for n=17 ± SEM. cAMP levels were monitored in both inactivated and ADP-activated platelets at the following time points: Day 0 (baseline), Day 14 (following AGE administration) and Day 28 (washout). Volunteers consumed 5mL/day of AGE for a total of 14 days. Administration of AGE significantly ($P<0.05$) increased the levels of intracellular cAMP in both the inactivated and activated platelet samples (Day 14) when compared to the levels measured at Day 0 and Day 28, respectively. There was no significant difference noted in intracellular cAMP levels between the inactivated and activated platelets for any of the days tested. Key: U – inactivated platelets, A –ADP activated platelets.

DAY 0		DAY 14		DAY 28	
U	A	U	A	U	A
0.61 ± 0.14	1.18 ± 0.19	3.59 ± 1.23	4.56 ± 1.17	1.1 ± 0.21	1.27 ± 0.2

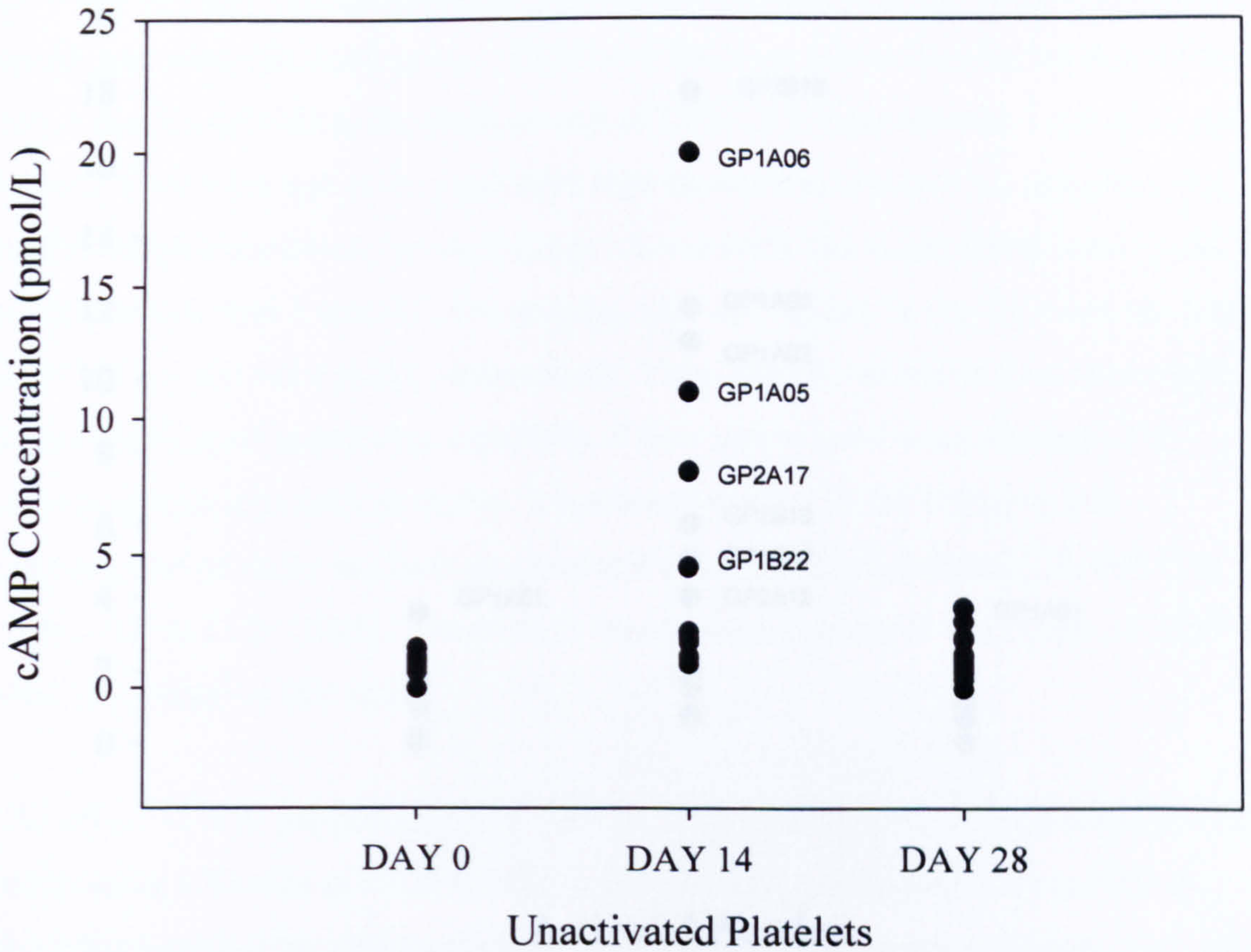

Figure 5.4. The effect of AGE on intraplatelet levels of cAMP *in vivo* in un-activated platelets. Platelet-rich plasma was removed from citrated blood on the following days during the supplementation trial: Day 0 (before AGE administration), Day 14 (following AGE administration for 14 days, 5 mL/day), and Day 28 (14 day washout period). Intraplatelet levels of cAMP were determined using an EIA assay kit. Data expressed shows the spread of cAMP for each of the participants on the various days (n=17). Individuals with high cAMP concentrations are indicated in the figure.

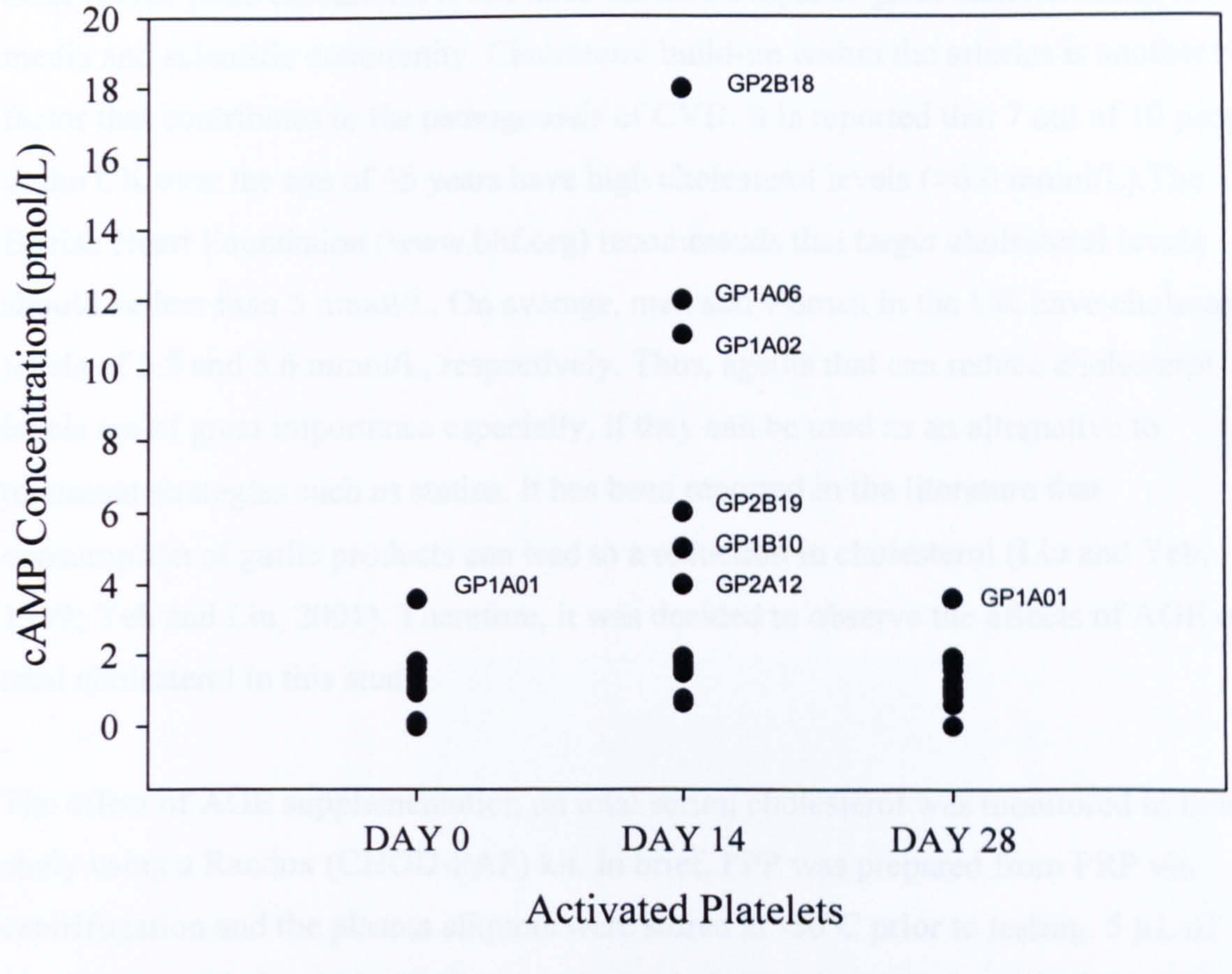

Figure 5.5. The effect of AGE on intraplatelet levels of cAMP *in vivo* in ADP-activated platelets. Platelet-rich plasma was removed from citrated blood on the following days during the supplementation trial: Day 0 (before AGE administration), Day 14 (following AGE administration for 14 days, 5 mL/day), and Day 28 (14 day washout period). Platelets were stimulated with the agonist ADP (8μmol/L) and the reaction was allowed to run for 5 min before termination by placing the samples on ice. Intraplatelet levels of cAMP were determined using an EIA assay kit. Data expressed shows the spread of cAMP for each of the participants on the various days (n=17). Individuals with high cAMP concentrations are indicated in the figure.

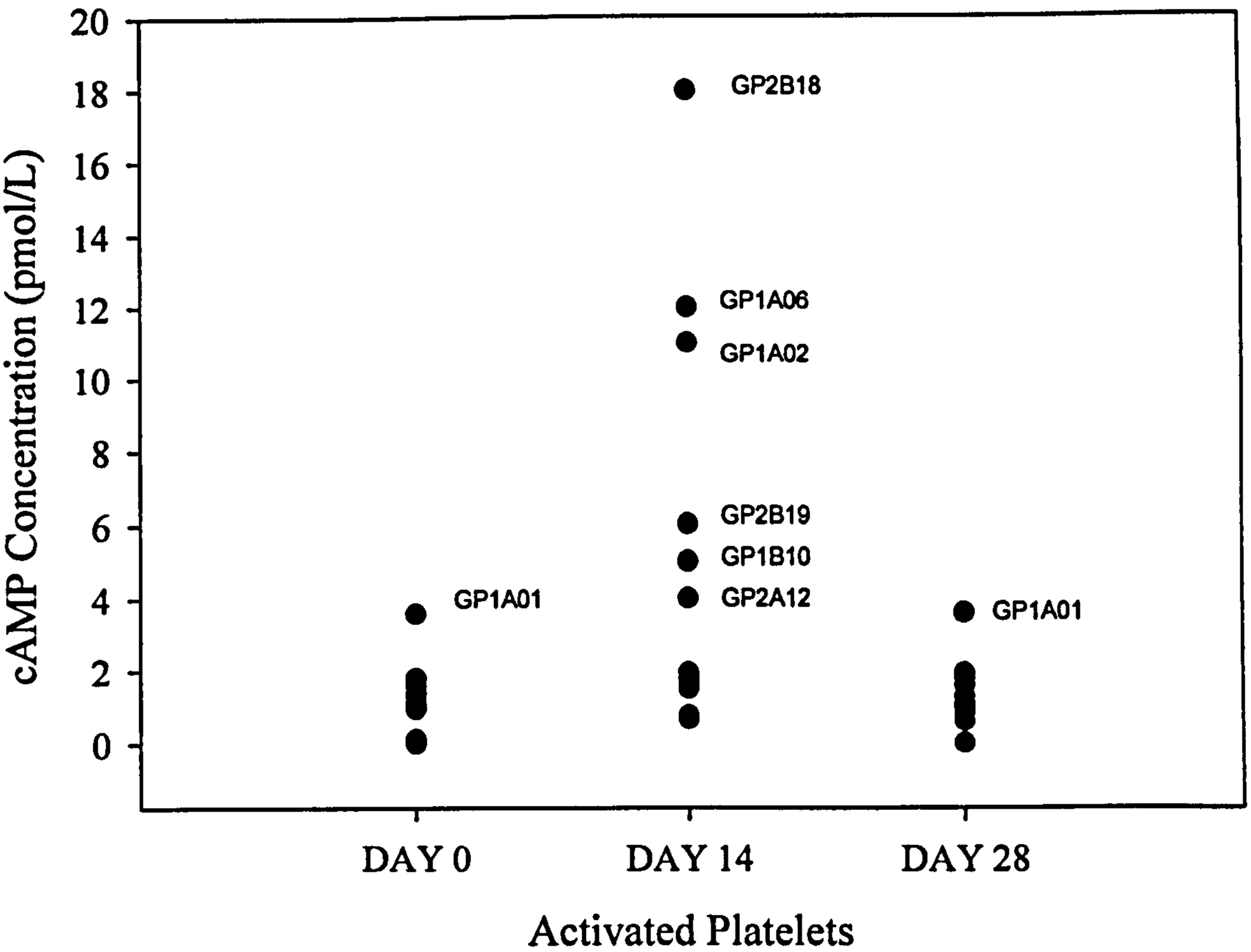

Figure 5.5. The effect of AGE on intraplatelet levels of cAMP *in vivo* in ADP-activated platelets. Platelet-rich plasma was removed from citrated blood on the following days during the supplementation trial: Day 0 (before AGE administration), Day 14 (following AGE administration for 14 days, 5 mL/day), and Day 28 (14 day washout period). Platelets were stimulated with the agonist ADP (8μmol/L) and the reaction was allowed to run for 5 min before termination by placing the samples on ice. Intraplatelet levels of cAMP were determined using an EIA assay kit. Data expressed shows the spread of cAMP for each of the participants on the various days (n=17). Individuals with high cAMP concentrations are indicated in the figure.

5.3.2.4. The effect of AGE on total cholesterol in vivo

Over recent years cholesterol levels have become a topic of great interest within the media and scientific community. Cholesterol build-up within the arteries is another risk factor that contributes to the pathogenesis of CVD. It is reported that 7 out of 10 people in the UK over the age of 45 years have high cholesterol levels (>6.0 mmol/L).The British Heart Foundation (www.bhf.org) recommends that target cholesterol levels should be less than 5 mmol/L. On average, men and women in the UK have cholesterol levels of 5.5 and 5.6 mmol/L, respectively. Thus, agents that can reduce cholesterol levels are of great importance especially, if they can be used as an alternative to treatment strategies such as statins. It has been reported in the literature that consumption of garlic products can lead to a reduction in cholesterol (Liu and Yeh, 1999; Yeh and Liu, 2001). Therefore, it was decided to observe the effects of AGE on total cholesterol in this study.

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The effect of AGE supplementation on total serum cholesterol was monitored in this study using a Randox (CHOD-PAP) kit. In brief, PPP was prepared from PRP via centrifugation and the plasma aliquots were stored at -80°C prior to testing. 5 µL of plasma was added to 250 µL of reagent in a microtitre plate and the optical density was measured at 500 nm after a 10 min incubation period at room temperature. All optical densities for each sample were done in duplicate. The standard (2 mg/mL ≈ 5.17 mmol/L) provided with the kit was measured in triplicate and the optical density was used to determine the concentration of cholesterol (mmol/L) contained with the plasma aliquot. Desirable levels of blood cholesterol are < 5.17 mmol/L, values over 6.2 mmol/L are considered undesirable.

Figure 5.6 shows that the total serum cholesterol level for the group at Day 0 (baseline) was 4.32 ± 0.19 mmol/L. Following AGE administration for 14 days (Day 14) the level was reduced to 4.13 ± 0.19 mmol/L, and further reduced at Day 28 (washout) to 4.04 ± 0.22 mmol/L. Even though a reduction was observed it was not deemed significant (P>0.05). Hence, AGE did not reduce total serum cholesterol in this clinical trial. Individual results (Appendix VIII) show that the cholesterol levels ranged from 2.6 – 5.6 mmol/L, indicating that all the subjects tested were normolipidemic.

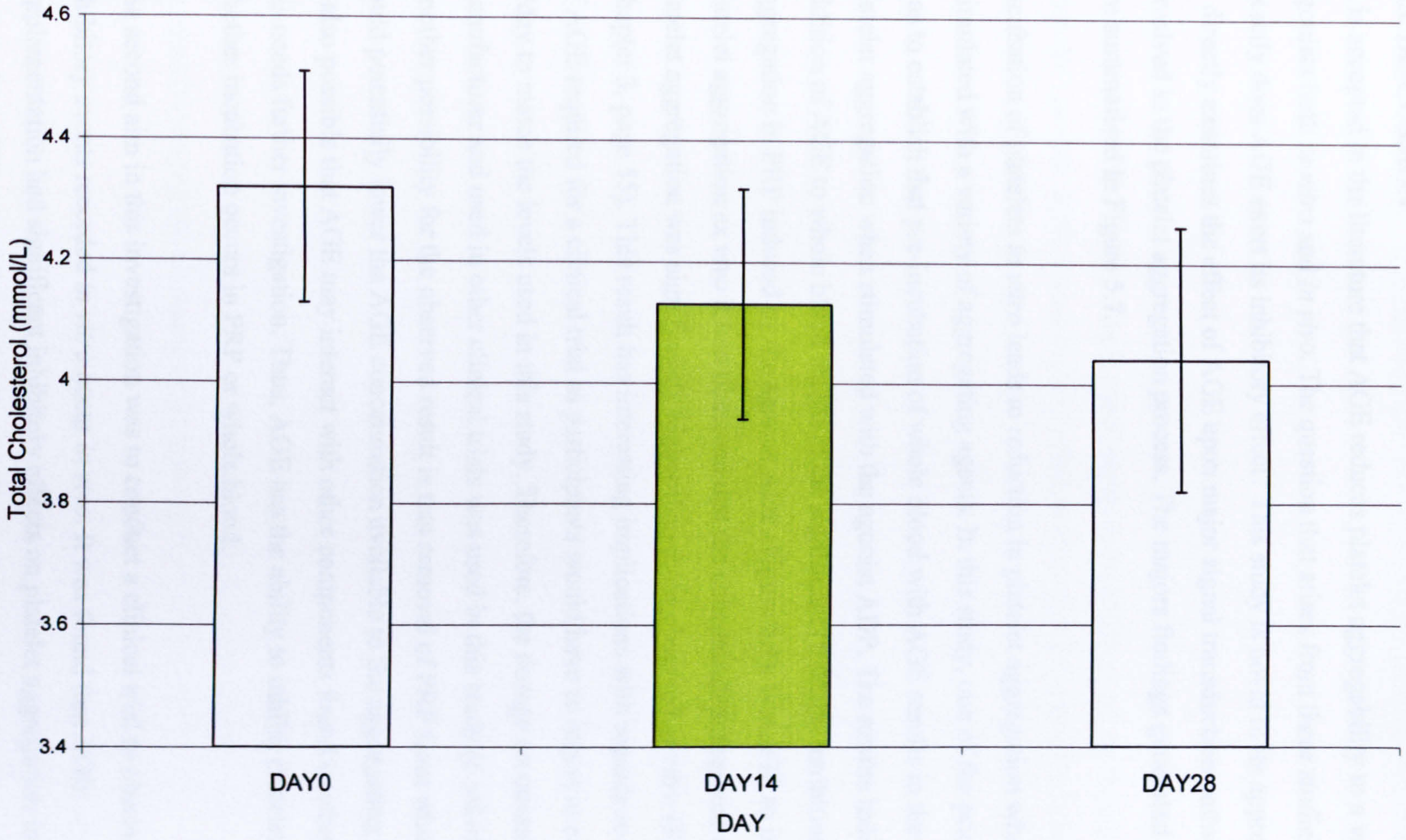

Figure 5.6. The effect of AGE on total cholesterol *in vivo.* Plasma samples were prepared by centrifugation of whole blood and aliquots were kept at -80°C prior to cholesterol analysis. Total cholesterol was monitored at Day 0, Day 14 (following AGE ingestions) and at Day 28 following a 14 day washout period. No statistical significance ($P>0.05$) noted between any of the Days.

5.5. DISCUSSION

It is accepted in the literature that AGE reduces platelet aggregability to a variety of agonists both *in vitro* and *in vivo*. The question that arises from these studies is how exactly does AGE exert its inhibitory effect? This study is novel in its approach in that it directly examines the effect of AGE upon major signal transduction pathways involved in the platelet aggregation process. The majors findings generated in this study are summarised in Figure 5.7.

Incubation of platelets *in vitro* leads to reduction in platelet aggregation when stimulated with a variety of aggregating agents. In this study, one of the primary aims was to establish that pre-incubation of whole blood with AGE results in the inhibition of platelet aggregation when stimulated with the agonist ADP. The results indicate that addition of AGE to whole blood results in the significant ($P<0.05$) inhibition of platelet aggregation in PRP induced by the agonist ADP (Figure 5.1). For AGE to inhibit platelet aggregation *ex vivo* it was observed that the concentration required to reduce platelet aggregation was significantly higher than those observed *in vitro* (Figure 3.1, Chapter 3, page 55). This result has interesting implications with regards to the dosage of AGE required for a clinical trial as participants would have to ingest in excess of 0.5 L/day to match the levels used in this study. Therefore, the dosage recommended by the manufacturer and used in other clinical trials was used in this study (5 mL/day). Another possibility for the observed result is that removal of PRP from whole blood could potentially lower the AGE concentration available to the aggregating platelets. It is also possible that AGE may interact with other components found in whole blood, this needs further investigation. Thus, AGE has the ability to inhibit platelet aggregation whether incubation occurs in PRP or whole blood.

The second aim in this investigation was to conduct a clinical trial to observe if the inhibitory events recorded *in vitro* occur *in vivo*. It was found that AGE supplementation had significant inhibitory effects on platelet aggregation induced by the agonists ADP, adrenaline and collagen (Figure 5.2). Similar studies using AGE have also shown that following AGE ingestion for a set time period resulted in inhibition of agonist-induced platelet aggregation (Rahman and Billington 2000; Steiner and Lin 1998; Steiner *et al.*, 1996; Steiner and Lin 1994; Steiner and Li 2001). The major difference between this study and others conducted with AGE is the time period for

AGE administration. As Steiner and Lin (1994) conducted their study over a 6 month period whereas, Rahman and Billington (2000) ran their study for 13 weeks. Does the length of the administration period play a role in the effectiveness of AGE to reduce platelet aggregation?

The results from the clinical trial show that following AGE administration for 14 days the level of platelet aggregation is significantly reduced when aggregation is initiated with the agonists ADP, adrenaline and collagen (Figure 5.2). It was also observed that the levels of platelet aggregation after washout (Day 28) were comparable to those at Day 0 (baseline). Thus, it can be suggested that AGE is effective in reducing platelet aggregation stimulated by the agonists ADP, adrenaline and collagen *in vivo*. The disappointing result was with the agonist AA, as no significant inhibition was observed following AGE supplementation. The results obtained for AA-induced platelet aggregation showed considerable variation between the individuals tested (Appendix VIII). One such reason for this occurrence is that certain members of the study group may have taken medications such as aspirin and NSAIDs, and failed to report this during the study. It is well documented in the literature that aspirin reduces the efficacy of platelet aggregation via the inhibition of the enzyme COX. This enzyme is involved in AA metabolism and the subsequent production of metabolites such as TXA_2, an important mediator in the amplification of the aggregation response, and inhibition of this enzyme stops platelet aggregation. As mentioned previously in this study, aspirin has no effect upon aggregation initiated with ADP, indicating a TXA_2-independent pathway is involved in platelet aggregation stimulated with this agonist. The same can be said for the agonist adrenaline, as both receptors for these agonists are linked to G-proteins and alternative stimulatory signalling pathways independent of AA metabolism are stimulated upon agonist-receptor interaction.

In vitro (Chapter 4, Figure 4.19, page 131) testing showed that pre-incubation of PRP with AGE resulted in a significant decrease in platelet aggregate formation via fibrinogen binding. Therefore, it was decided to observe the effect of AGE on this process *in vivo*. Figure 5.3 shows that AGE supplementation has no significant inhibitory effect upon GPIIb/IIIa binding and the subsequent formation of stable platelet aggregates in both the activated and inactivated platelet (Figure 5.4) groups as examined by whole blood flow cytometry. This result differs greatly from those obtained during *in*

vitro testing (Chapter 4, Figure 4.19, page 131) suggesting that AGE must exert its inhibitory affect at a different point(s) in the signalling pathway prior or proceeding to GPIIb/IIIa receptor activation *in vivo*. Another possible explanation for the observed result is that the fibrinogen receptor may have undergone a conformational change (receptor changed from inactive to active state) which resulted in a considerable number of platelets being positive for the antibody PAC-1 in the activated platelet group, as shown in Figure 5.4. The antibody PAC-1 acts by binding to a specific site on the GPIIb/IIIa receptor once the receptor has undergone conformational change. It is possible that platelets may have changed shape following agonist stimulation and were prevented from aggregating by other factors involved in the aggregatory process. It is known from the aggregation experiments that AGE does reduce agonist-induced platelet aggregation *in vivo* (Figure 5.2, page 161). One possible explanation for this observed result is that AGE has to directly come into contact with the receptor in order to exert an inhibitory effect and that its is concentration-dependent (indirect – *in vivo* vs direct – *in vitro*).

Total serum cholesterol was monitored in this study. It was found that all the subjects who participated in the study were normolipidemic (<6.0 mmol/L). Following AGE consumption (Day 14) and after the washout period (Day 28) there was a decrease in the total serum cholesterol (Figure 5.6) however, this reduction was not deemed significant ($P>0.05$) following statistical analysis. These results are consistent with those presented by Rahman and Billington (2000), in that they too found no significant changes to total serum cholesterol following AGE administration. Studies where AGE has been shown to reduce total serum cholesterol (Lau *et al.* 1987; Yeh *et al.* 1995, 1997) were performed in patients who were hyperlipidemic. Thus, in this study it would not be expected to observe significant reductions in total cholesterol following AGE consumption as all the patients tested were apparently healthy individuals who were normolipidemic. Another trial would need to be conducted to include those in the population with elevated levels of total serum cholesterol.

The most important result generated in this study is that AGE supplementation increases the intraplatelet levels of cAMP in both inactivated and ADP-activated platelets. This result validates the findings from the *in vitro* study (Chapter 4, section 4.2.3, pages 114-115) which also showed significant increases in cAMP subsequent to AGE treatment.

The levels recorded at Day 0 and Day 28 was very similar in both the inactivated and activated platelet groups. There was no difference between the inactivated and activated platelet groups. Collectively, the findings generated in this study were statistically significant ($P<0.05$) for all the volunteers tested. It is important to note that only certain individuals within the study were susceptible to the actions of AGE upon intraplatelet cAMP levels (23 % - inactivated, 35 % activated). The levels of cAMP for the individuals were extremely high compared to the remaining volunteers hence, the observed statistical significance. This result suggests that AGE is effective as long as it is continually being taken by the subject, and that 14 days is sufficient washout time period for removing the effects of thisAGE from the human circulatory system.

The results from this study suggest that inhibition of platelet aggregation does occur following AGE administration and that the degree of inhibition is individual-dependent. Also the biochemical signalling processes affected by AGE *in vivo* are subject to inter-individual variation.

The major problem encountered with this study is that the AGE samples were not taken at the same time everyday by all the participants. This was due largely in part to the intense flavour of the liquid extract. It is recommended by the manufacturer to take the liquid extract in orange juice or even tomato juice. Feedback from the volunteers implied that it was difficult to mask the flavour and that the only way in which it could be taken was in the evening with a savoury meal. One recommendation is that instead of taking the liquid AGE as it is, it would be better if it were placed into gel capsule like those used for cod liver oil, as more people would be willing to take the extract in this way. It is possible that many of the subjects were not very compliant throughout the study, although compliance was monitored through weekly doses being given to subjects. Also at the end of the trial volunteers were asked to return there liquid AGE containers. One way to measure compliance would be to monitor the amount of SAC in the subject's urine using HPLC as done by Dillon *et al.* (2002). However, for the purpose of this study it was impractical to measure SAC levels in urine. The sample collection and processing would have impeded on other experiments that needed to be conducted. It is also possible that more inhibition of platelet aggregation and the signalling pathways involved would have occurred if the study was carried out for a longer time period. Also a higher dose of AGE may have been more effective, as shown

in the *in vitro* platelet aggregation studies whereby, dose-dependent inhibition was observed. For the purpose of this study the recommended daily dose of AGE was utlised in order to show that ingestion over a short time period has beneficial effects with regards to platelet aggregation.

Another issue that affected the planning and execution of the study was technical difficulties. In that there was only one experimenter conducting all the experiments. This greatly affected the choice of experiments that were conducted. It would have been interesting to observe the *in vivo* effects of AGE upon calcium ion mobilisation within the platelet. The *in vitro* data generated from monitoring calcium ion moblisation (Chapter 4) looked very promising in that, AGE appeared to reduce the levels available to aggregating platelets when compared with the test controls. Also there is a definate link between cAMP levels in the platelet and available calcium ions. An increase in cAMP leads to a decrease in calcium that ultimately results in a reduction in the levels of platelet aggregation. If this study were to be conducted again it would be advisable to monitor both cAMP and calcium mobilisation simultaneously.

The sample size used in this study was sufficient enough to gather data for statistical purposes. However, future experiments should aim to increase this size and include those with pre-existing conditions such as hyperlipidemia to ensure that AGE does definitely reduce this *in vivo*. It should be noted that although more females (11) participated than males (6) in this study, there was no significant difference between their results for any of the experiments conducted (Appendix VIII). Also those who drank regularly were no different than those who did not. The smokers in the group when compared to the non-smokers had no differences either. To reiterate the primary variable under investigation in this study was the direct effect of AGE upon platelet function, and all volunteers who participated had normal platelet counts and platelet aggregate formation.

The results generated in this study should be seen as a recommendation for the use of AGE to protect against the onset of disease states such as atherosclerosis. AGE definitely has anti-platelet effects and with further investigation into its properties and effects on the signal transduction pathways in platelets it has the potential to be a preventative therapy in the fight against CVD.

5.4. SUMMARY OF RESULTS

- AGE inhibited platelet aggregation induced by ADP *ex vivo*.
- AGE inhibited agonist-induced platelet aggregation *in vivo* when stimulated by the agonists ADP, adrenaline and collagen.
- AGE did not suppress fibrinogen-receptor interaction to form a stable platelet aggregate *in vivo*.
- AGE increased the intraplatelet levels of cAMP *in vivo* in both inactivated and activated platelets.
- AGE does not have an effect upon the total serum cholesterol levels *in vivo* in normolipidemic subjects.

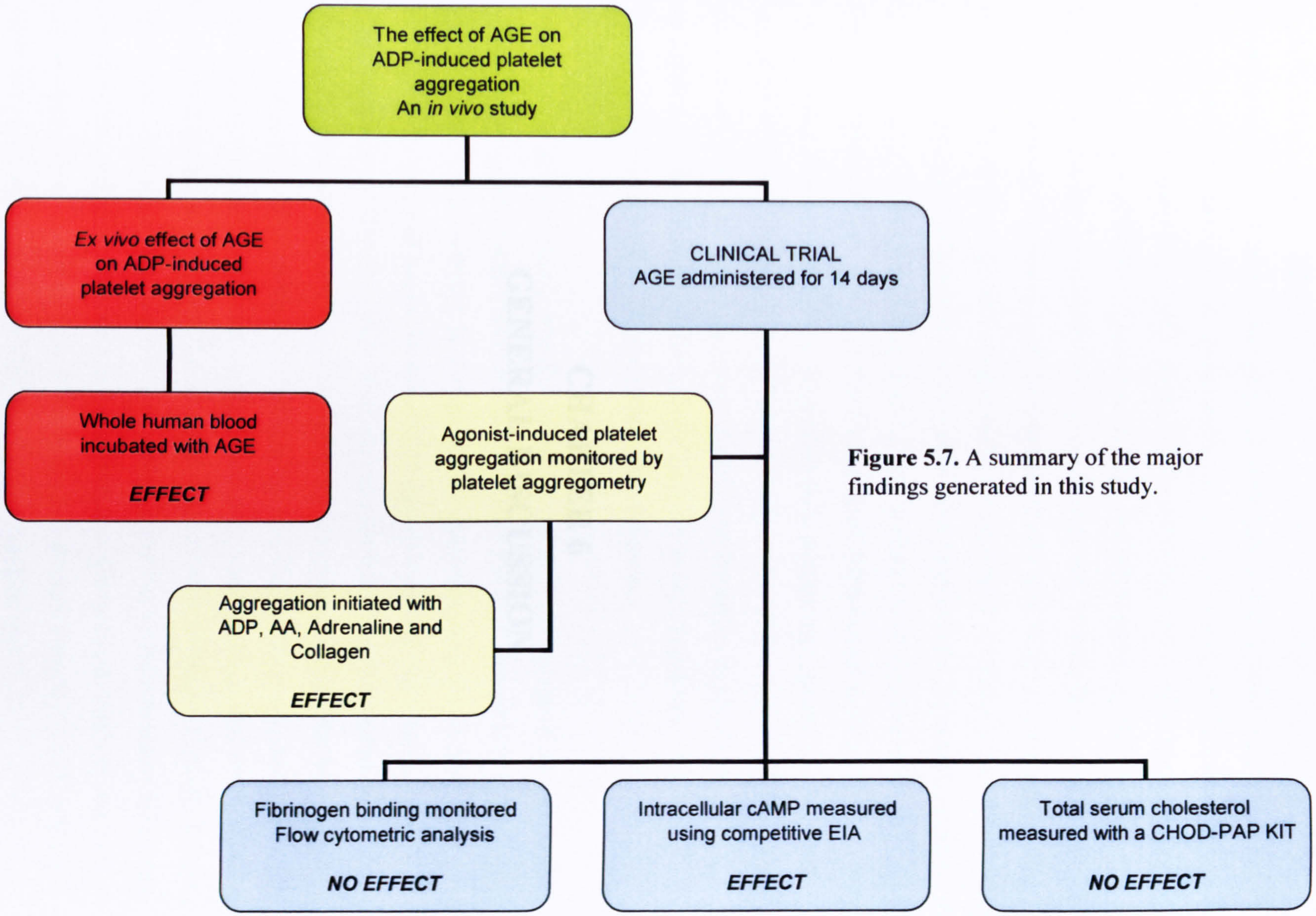

Figure 5.7. A summary of the major findings generated in this study.

CHAPTER 6

GENERAL DISCUSSION

6. GENERAL DISCUSSION

Herbal compounds such as garlic, ginger, kinetin, glucosamine, tomato extract, etc., through scientific testing have been found to possess cardioprotective properties (Bordia *et al.* 1978; Duttaroy *et al.* 2002; Fugh-Berman 2000; Hua *et al.* 2004; Innes *et al.* 2003; Osmont *et al.* 2003; Rahman and Billington 2000; Sheu *et al.* 2003; Son *et al.* 2004). These natural compounds reduce risk factors such as: a reduction in blood lipids and triglycerides, enhancement of circulation and reduction in blood pressure. Garlic, especially the dietary supplement aged garlic extract – AGE, (Kyolic®) has been shown to exhibit all these cardioprotective properties (www.kyolic.com). One such property of AGE is that it reduces platelet aggregation both *in vitro* and *in vivo* (Rahman and Billington 2000; Steiner and Lin 1994; Steiner *et al.* 1996 Steiner and Lin 1998; Steiner and Li 2001). The exact nature of this inhibition of platelet aggregation (mechanistic action of AGE on platelet cell signalling) with respect to AGE is unknown. Thus, the primary aim of this investigation was to elucidate an inhibitory mechanism(s) of AGE on agonist (ADP)-induced platelet aggregation both *in vitro* and *in vivo*. The major findings generated in this study are presented in Figure 6.1.

This study is novel in that; it actually monitored the biochemical signalling events that occurred during platelet aggregation both *in vitro* and *in vivo*. The platelet signalling pathways that were monitored in this study were chosen because they have been highlighted as potential targets from research already conducted on other garlic preparations in the literature, these biochemical events include: the formation of thromboxanes (Dimitrov and Bennick 1997; Harenburg *et al.* 1998; Makheja *et al.* 1980; Moon *et al.* 2000; Srivastava 1984; Srivastava 1993); phospholipase activity and lipoxygenase products (Ali 1995; Apitz-Castro *et al.* 1983; Liu and Yeh 1999) ; calcium mobilisation (Park *et al.* 2004; Qi *et al.* 2000; Shah *et al.* 1999) and GPIIb-IIIa (O'Neill *et al.* 2000; Tapiero *et al.* 2004) receptor activity. Another reason for using the chosen signalling events is that there are medicines which target these pathways to prevent platelet aggregation currently used in the clinic as treatment for CVD (COX activity, ADP receptor antagonists and GPIIb-IIIa receptor antagonism).

Comparisons of the *in vitro* and *in vivo* data generated in the study demonstrate that AGE does indeed inhibit platelet aggregation induced by a variety of agonists (Chapters

3 & 5, respectively). The results generated are consistent with those presented by other scientific studies that have monitored the effect of AGE on platelet function both *in vitro* and *in vivo* (Rahman and Billington 2000; Steiner and Lin 1994; Steiner *et al.* 1996; Steiner and Lin 1998; Steiner and Li 2001). *In vitro* testing (Chapter 4) showed that AGE targeted the following biochemical signalling pathways to inhibit platelet aggregation induced with the agonist ADP: calcium mobilisation, fibrinogen binding and, intraplatelet cAMP levels were increased/ decreased significantly. *In vivo* (Chapter 5) testing differed greatly from the *in vitro* work in that; the only biochemical pathway to be affected by AGE was an increase to the levels of intraplatelet cAMP to inhibit platelet aggregation induced with ADP. This observation may be dependent upon the agonist (ADP) used to initiate platelet aggregation and the signalling pathways associated with the ADP receptor that maintain the aggregatory response.

The signalling events that were monitored in this study occur downstream of receptor-agonist interaction therefore, it is important not to rule out the possibility that AGE acts directly at the receptor level for each agonist as the *in vitro* studies show that AGE inhibits a wide range of platelet agonists. Future studies could look at the signalling events associated with other agonists such as collagen, thrombin, adrenaline etc and the effect AGE has. Does AGE target points in the pathway common to all forms of agonist stimulation as shown with ADP? Also, initial studies monitoring proteolytic activity in AGE showed that it is possible that AGE does contain proteases following the outcome of the Azocasein assay. Therefore, before the idea is dismissed that AGE does not act at the receptor level for each agonist more experimentation is required. As AGE appears to be in possession of components which may exert their effects at various stages involved in the process of platelet aggregation. Individual components isolated from AGE (Chapter 3) also show that the antiplatelet potential of AGE cannot be predicted by organosulphur content alone and that the entire extract is required to exert any kind of inhibitory effect. For this reason it is impossible to compare the actions of AGE to other garlic preparations in that no two preparations contain the same mixture of ingredients. Also, the levels of certain constituents i.e. organosulphur compounds, oil-soluble compounds, carbohydrates, saponins etc differ in concentration when compared to AGE which contains compounds not found in other garlic supplements (Kasuga *et al.* 2001).

This study shows that AGE as a dietary supplement (clinical trial data presented in chapter 5) does have health benefits in that it reduces platelet aggregability both *in vitro* and *in vivo*. Therefore, AGE would be suitable as a preventative treatment for the disease state CVD. However, scientific testing is required with AGE and its interatctions with established prescribed medications such as warfarin. Is it safe to take AGE alongside other medications used in the treatment of CVD? There have been instances with other health food supplements interacting in an adverse manner with prescribed medication. This needs to be addressed further by conducting both *in vitro* and *in vivo* testing.

In conclusion, this study has shown that AGE is effective in inhibiting agonist-induced platelet aggregation both *in vitro* and *in vivo*. The principal mechanism found in this study for this observed inhibition both *in vitro* and *in vivo* appears to be via an increase to the intraplatelet levels of cAMP.

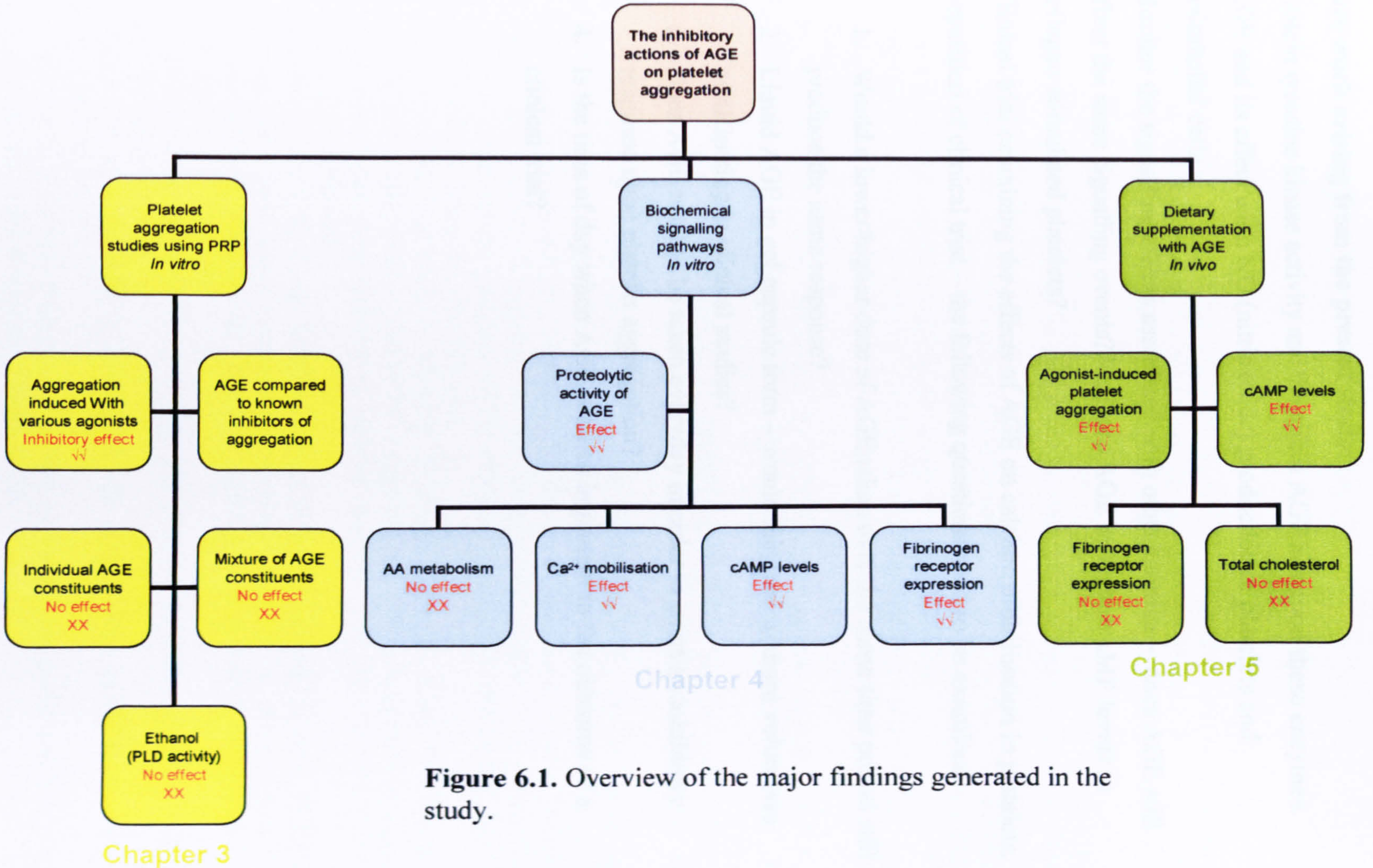

Figure 6.1. Overview of the major findings generated in the study.

6.1. Future work arising from the present study:

- Protein tyrosine kinase activity and the effect AGE has upon these enzymes.
- AGE and its effect upon NO (nitric oxide) production in platelets and endothelial cells.
- Monitor the signalling effects associated with other agonists – does AGE still affect the same signalling events? I.e. Does AGE increase cAMP levels in collagen-stimulated platelets?
- Clinical trial examining the effects of AGE on calcium mobilisation in platelets.
- Repetition of clinical trial – the following questions need to be examined:

1. Would a lower/higher dose of AGE taken over the same time period still produce the same response?
2. Liquid AGE in gel capsule form – would this lead to more volunteers participating in clinical studies?
3. Does AGE need to be taken everyday in order to exert an inhibitory response upon platelet aggregation?
4. Is the time of day when AGE is taken important to the outcome of a clinical trial?

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APPENDICES

APPENDIX I

Participant consent form

LIVERPOOL JOHN MOORES UNIVERSITY
FORM OF CONSENT TO TAKE PART AS A SUBJECT IN A MAJOR PROCEDURE OR RESEARCH PROJECT

Title of project/procedure: **Mechanisms of Inhibition of Platelet Aggregation by Aged Garlic Extract (Kyolic®)**

Voluntary donation of a 20 mL blood sample. To be taken via venepuncture of the median cubital vein by Gillian Allison, a PhD researcher in the School of Biomolecular Sciences. The platelets will be removed from whole blood and used to assess the inhibitory actions of AGE on platelet function.

I, ... agree to take part in
(Subjects full name)*

the above named project/procedure, the details of which have been fully explained to me and

described in writing.

Signed ..... Date.....
(Subject)

I, ...**Gillian Allison**..... certify that the details of this
(Investigators full name)*

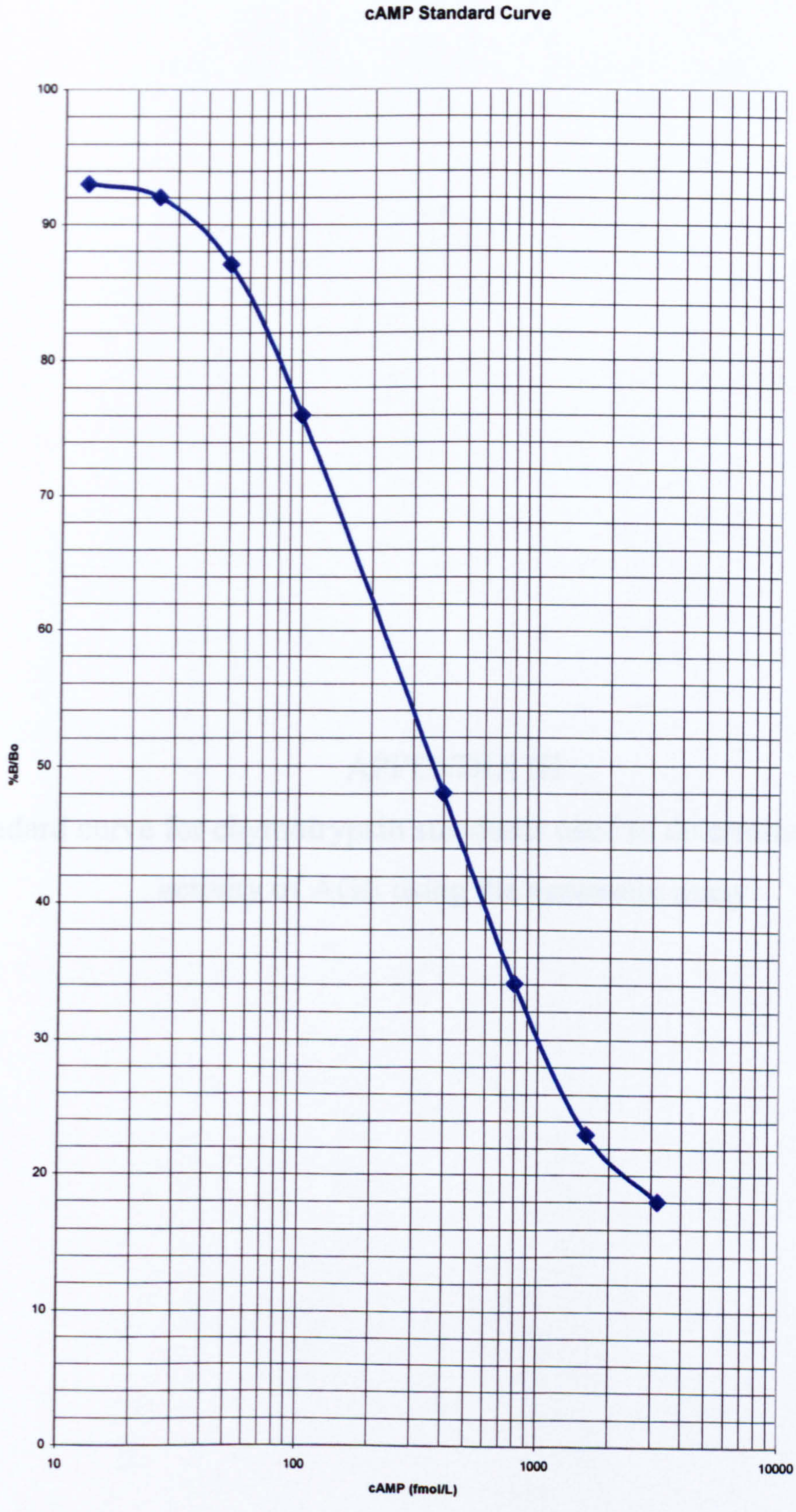
project/procedure have been fully explained and described in writing to the subject named above and

have been understood by him/her.

Signed ..... Date.....
(Investigator)

APPENDIX II

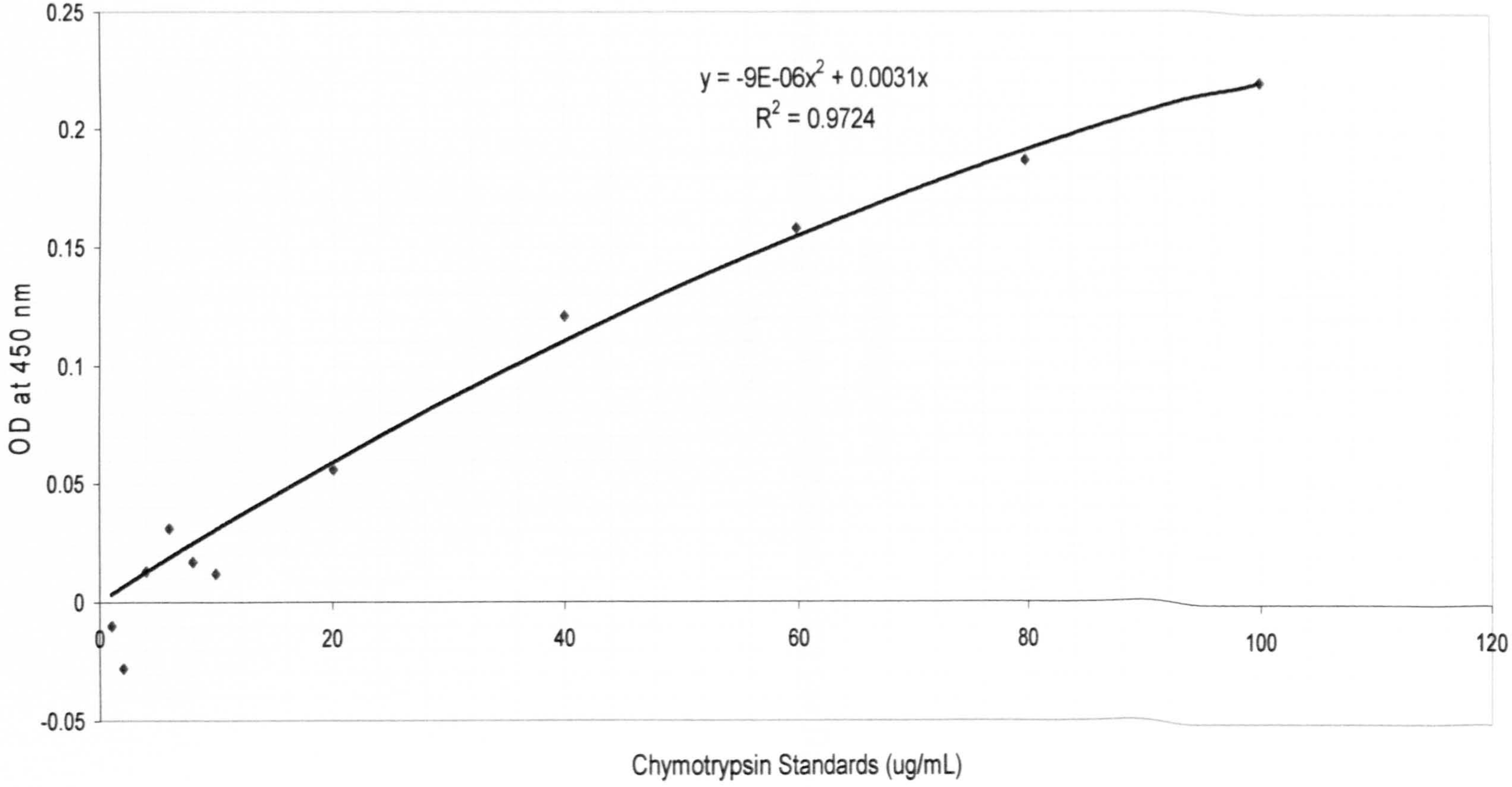
Standard curve for intraplatelet cAMP determination

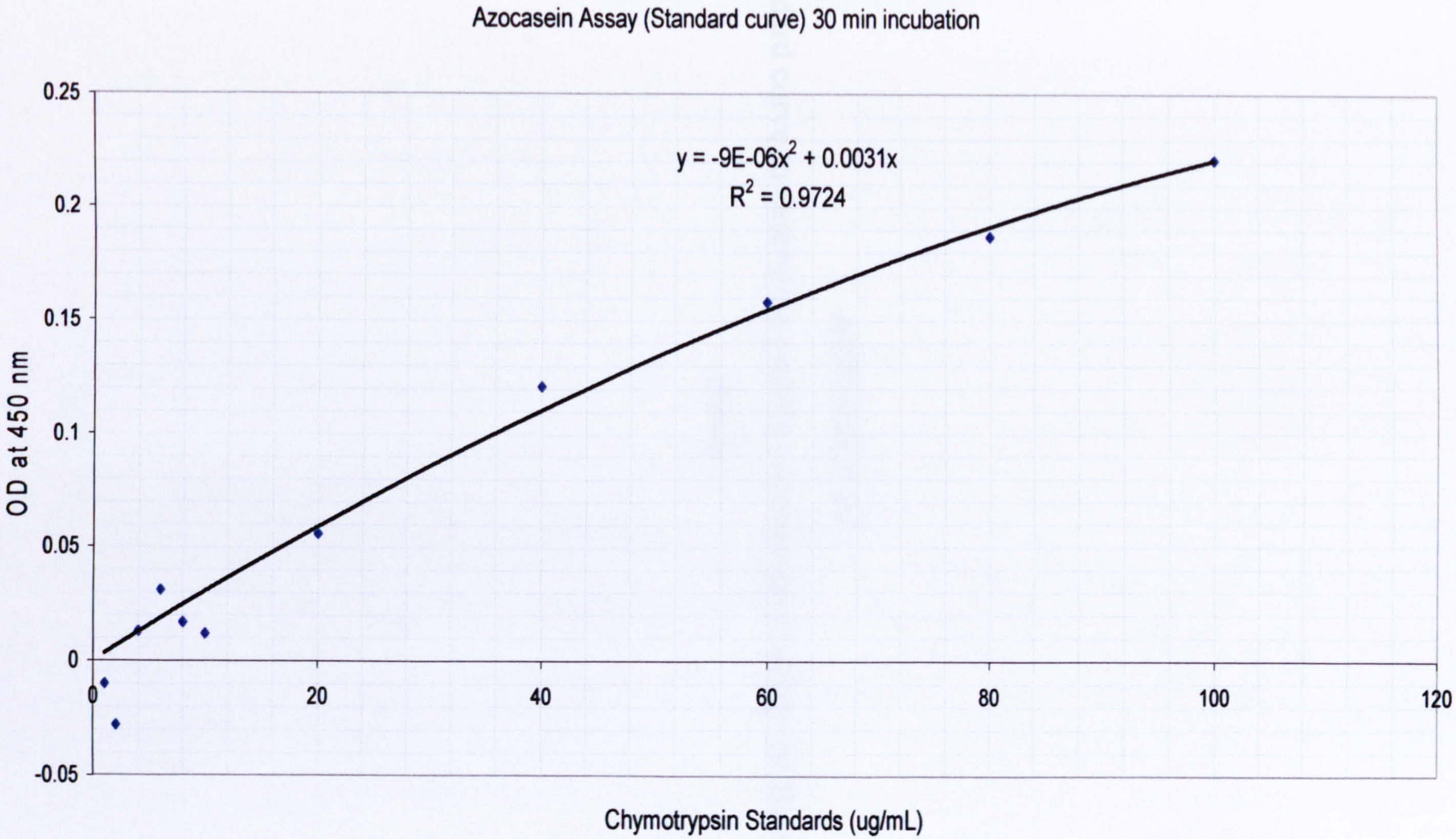

cAMP Standard Curve
100
90
80
70
60
50
40
30
20
10
0
%B/Bo
%B/Bo
10
100
1000
10000
cAMP (fmol/L)

APPENDIX III

Standard curve for chymotrypsin standards used to determine proteolytic activity of AGE using the azocasein assay

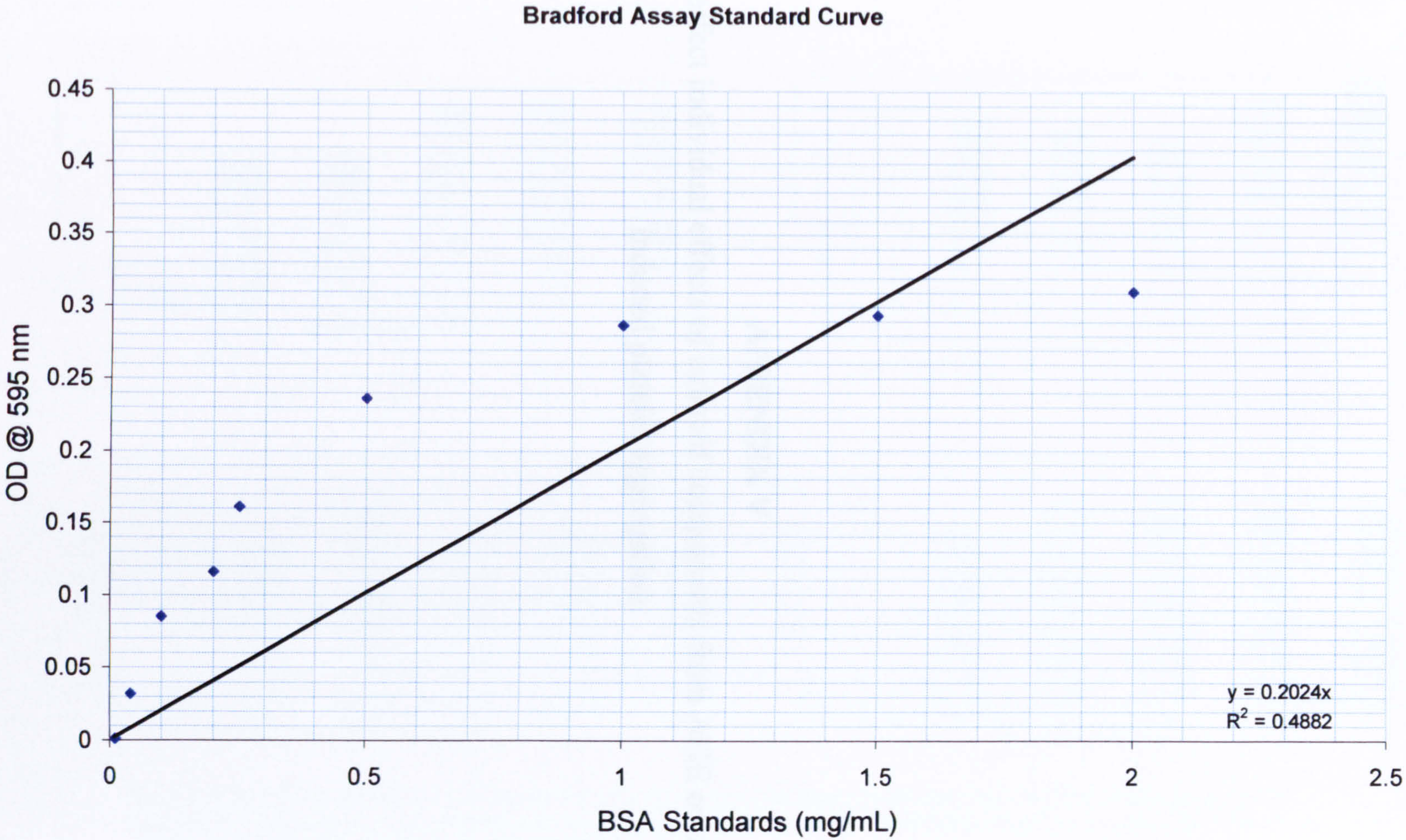
Azocasein Assay (Standard curve) 30 min incubation




Azocasein Assay (Standard curve) 30 min incubation
y = -9E-06x2 + 0.0031x
R2 = 0.9724
OD at 450 nm
0.25
0.2
0.15
0.1
0.05
0
-0.05
0
20
40
60
80
100
120
Chymotrypsin Standards (ug/mL)

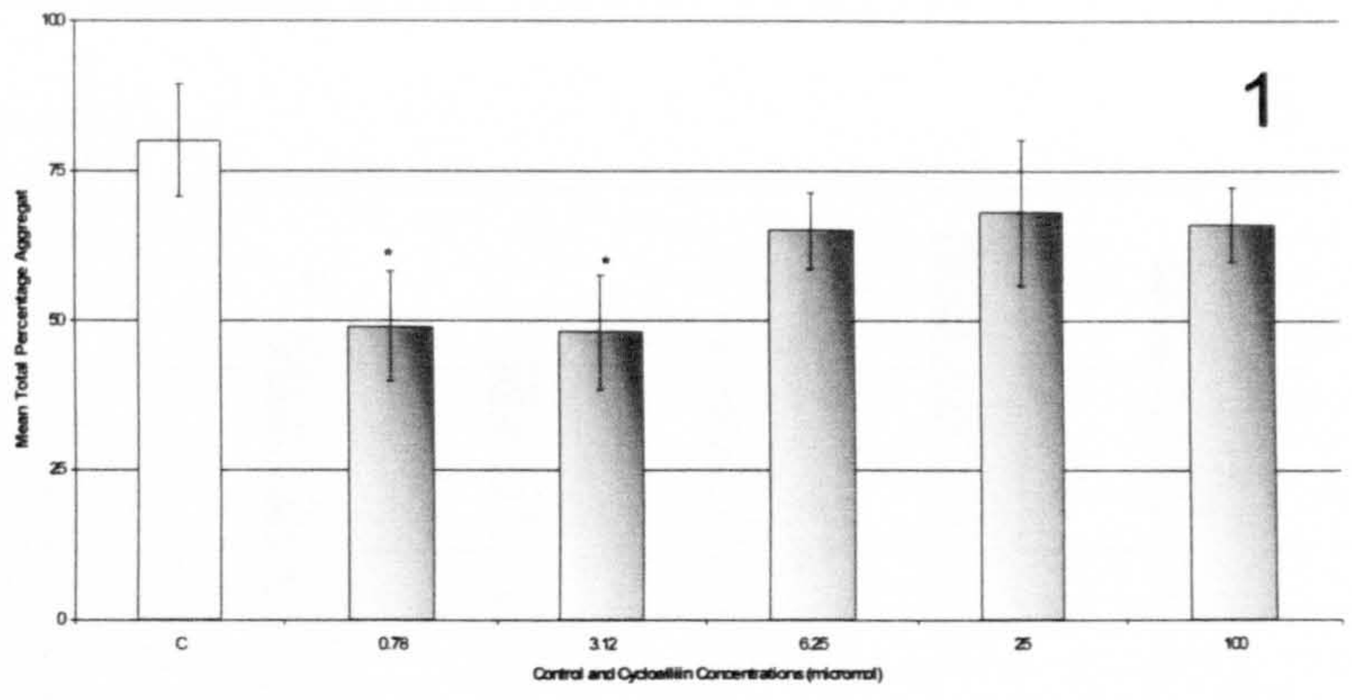
APPENDIX IV

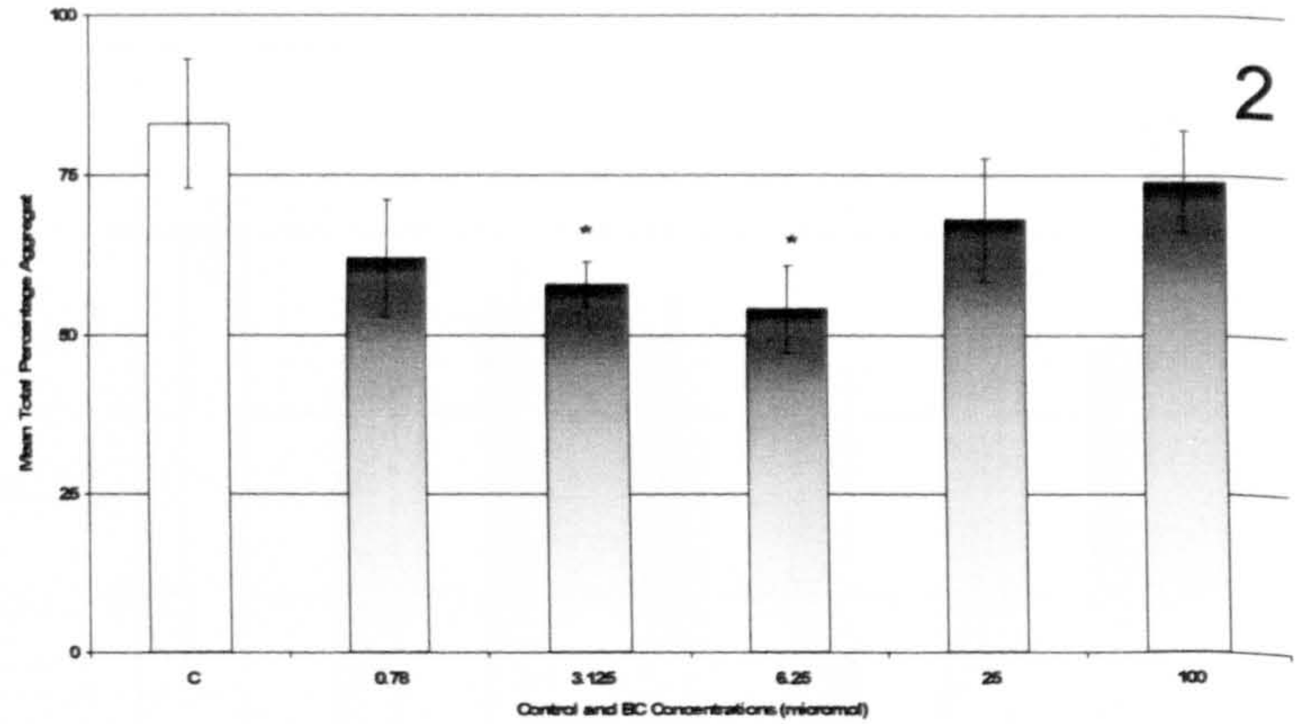
Standard curve to determine protein concentration using the Bradford Assay

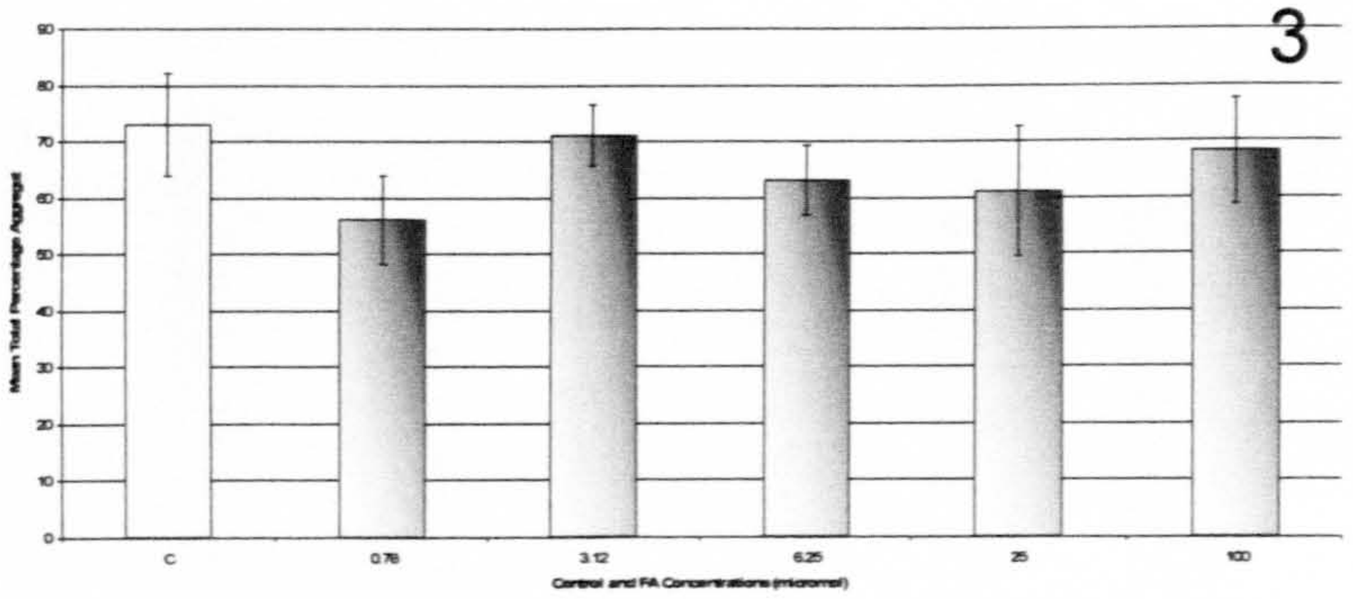

Bradford Assay Standard Curve
OD @ 595 nm
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.5
1
1.5
2
2.5
BSA Standards (mg/mL)
y = 0.2024x
R2 = 0.4882

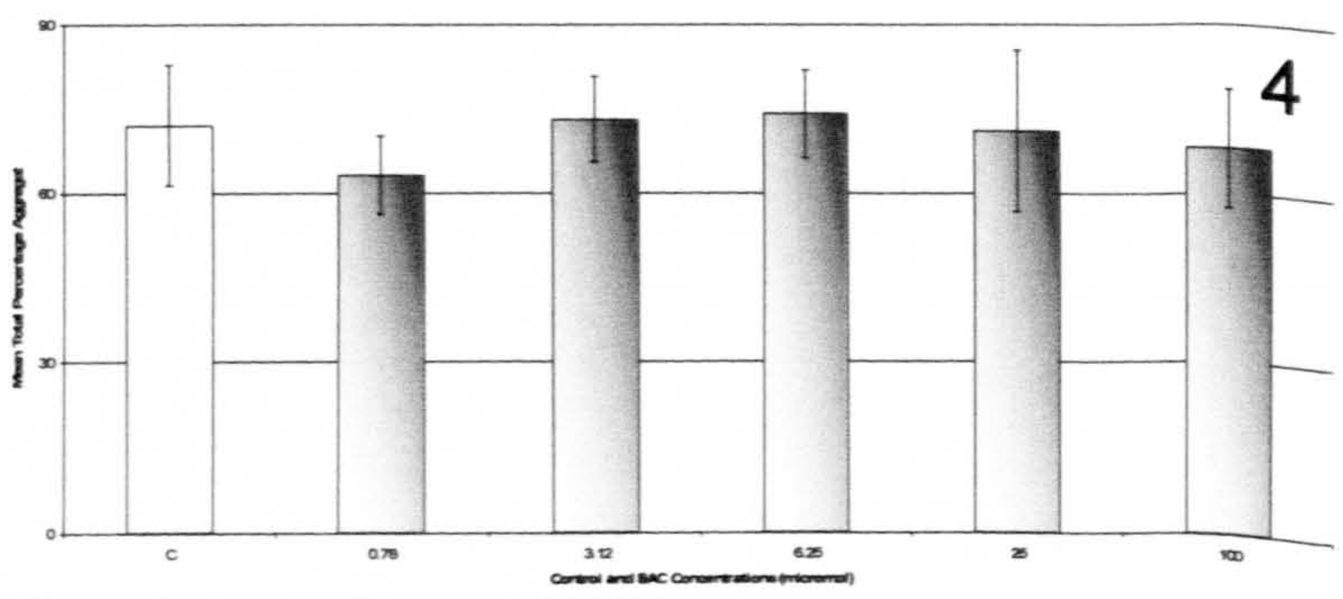
APPENDIX V

The effect individual effects of isolated components from AGE on ADP-induced platelet aggregation.

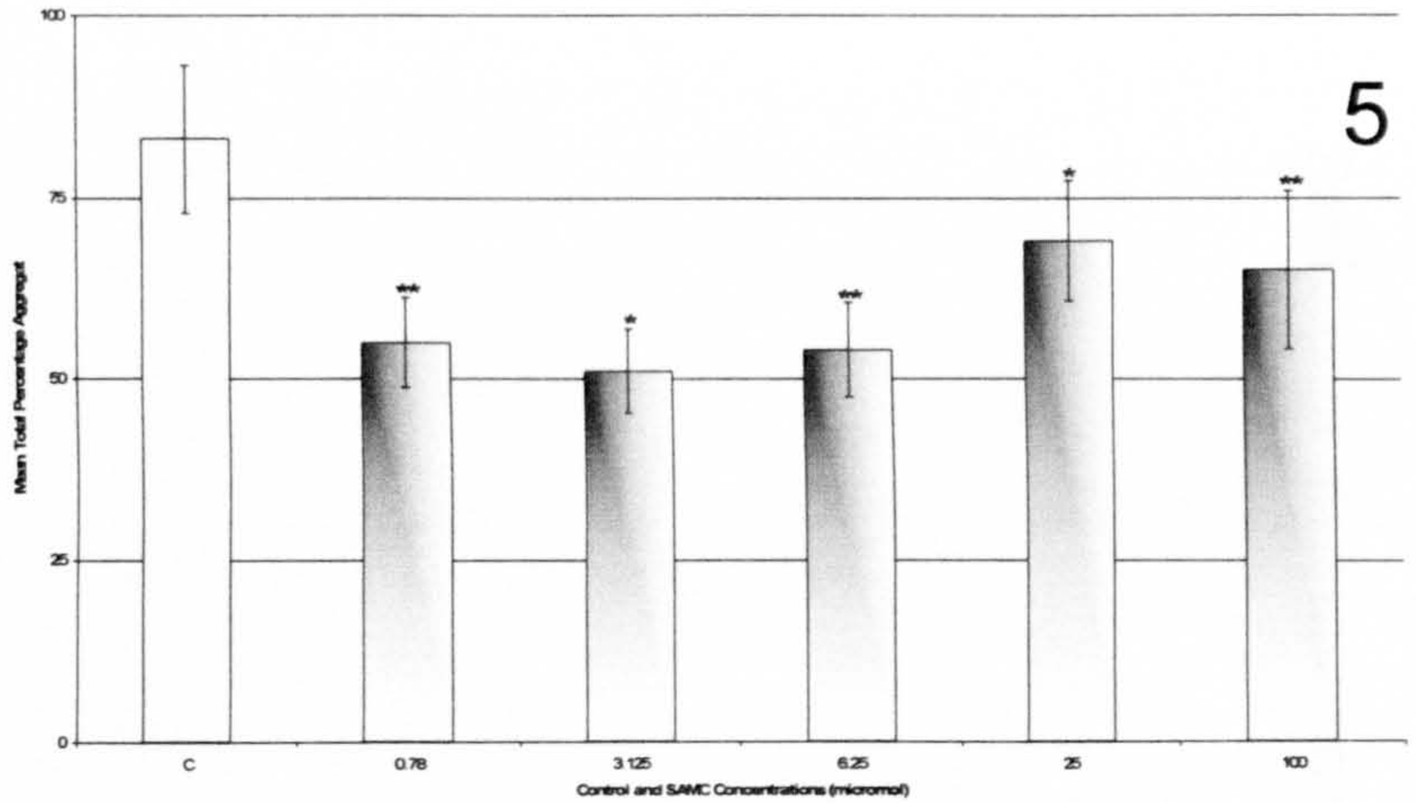


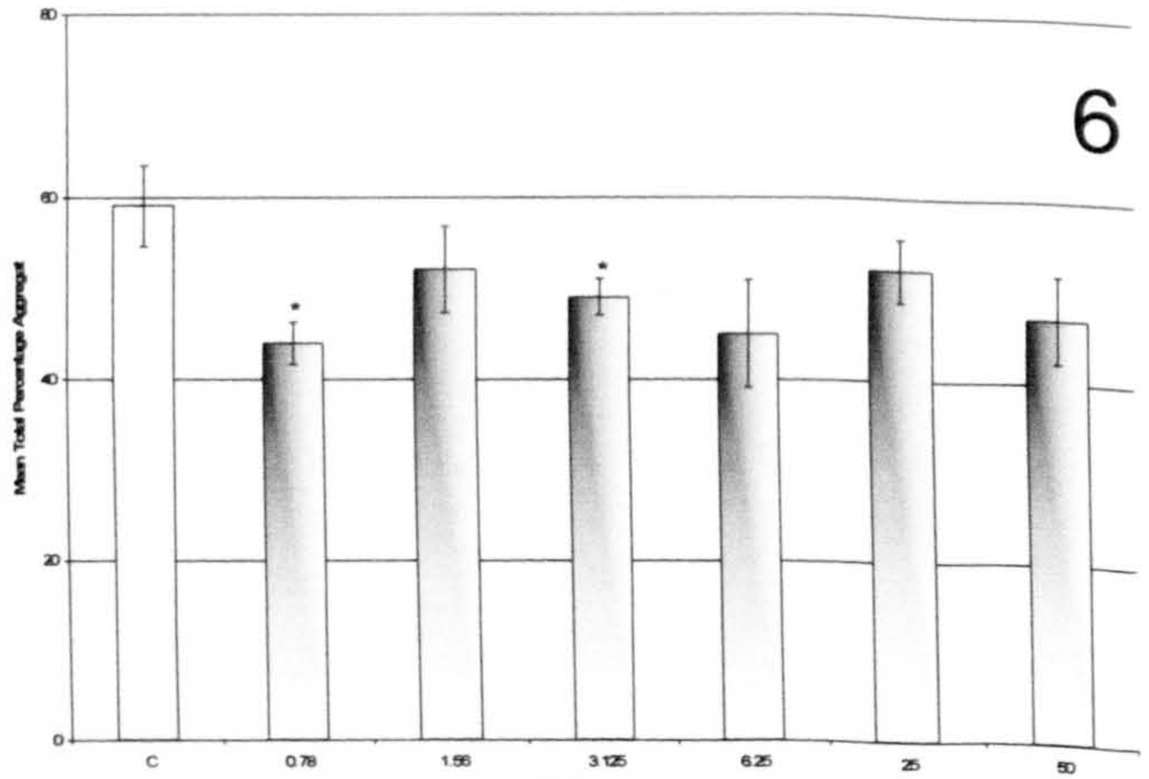


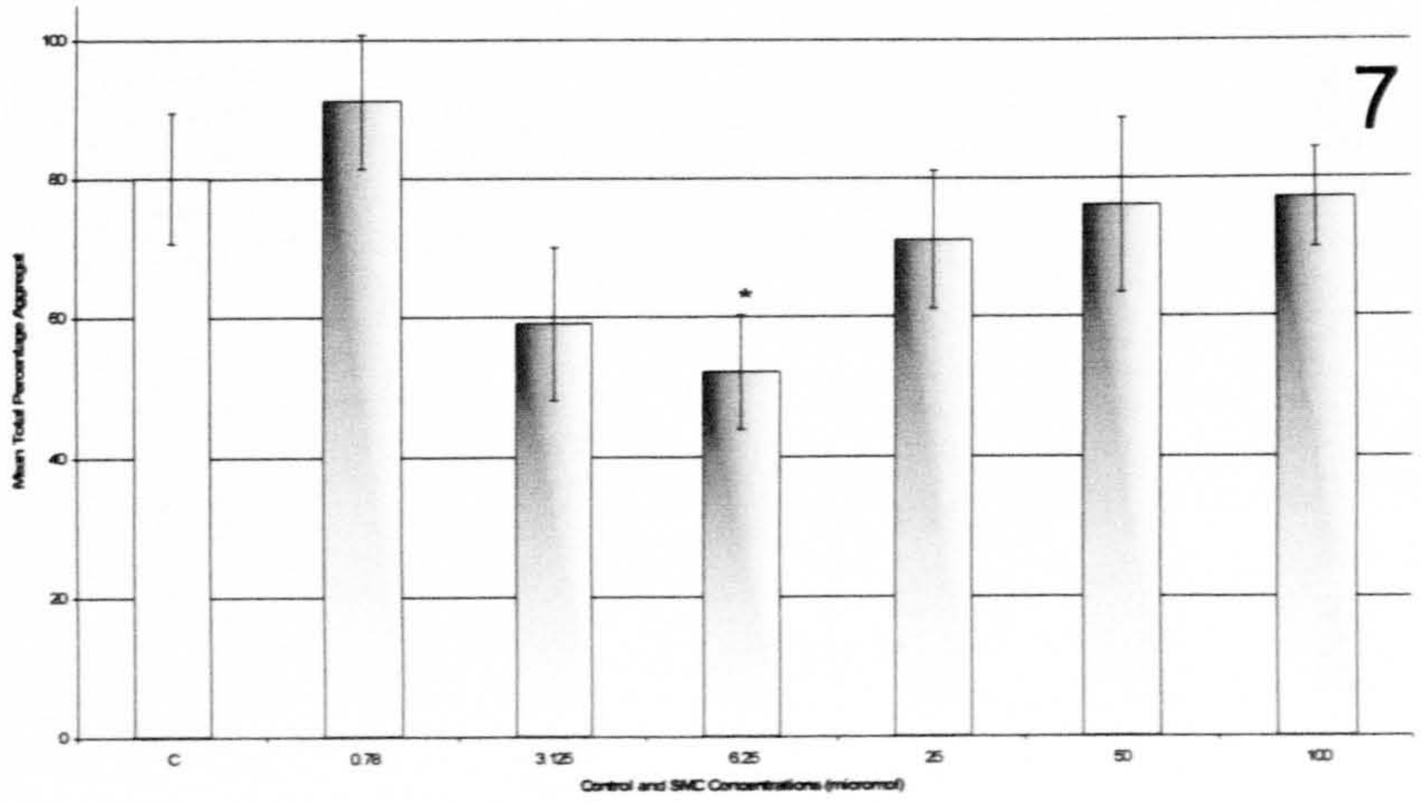


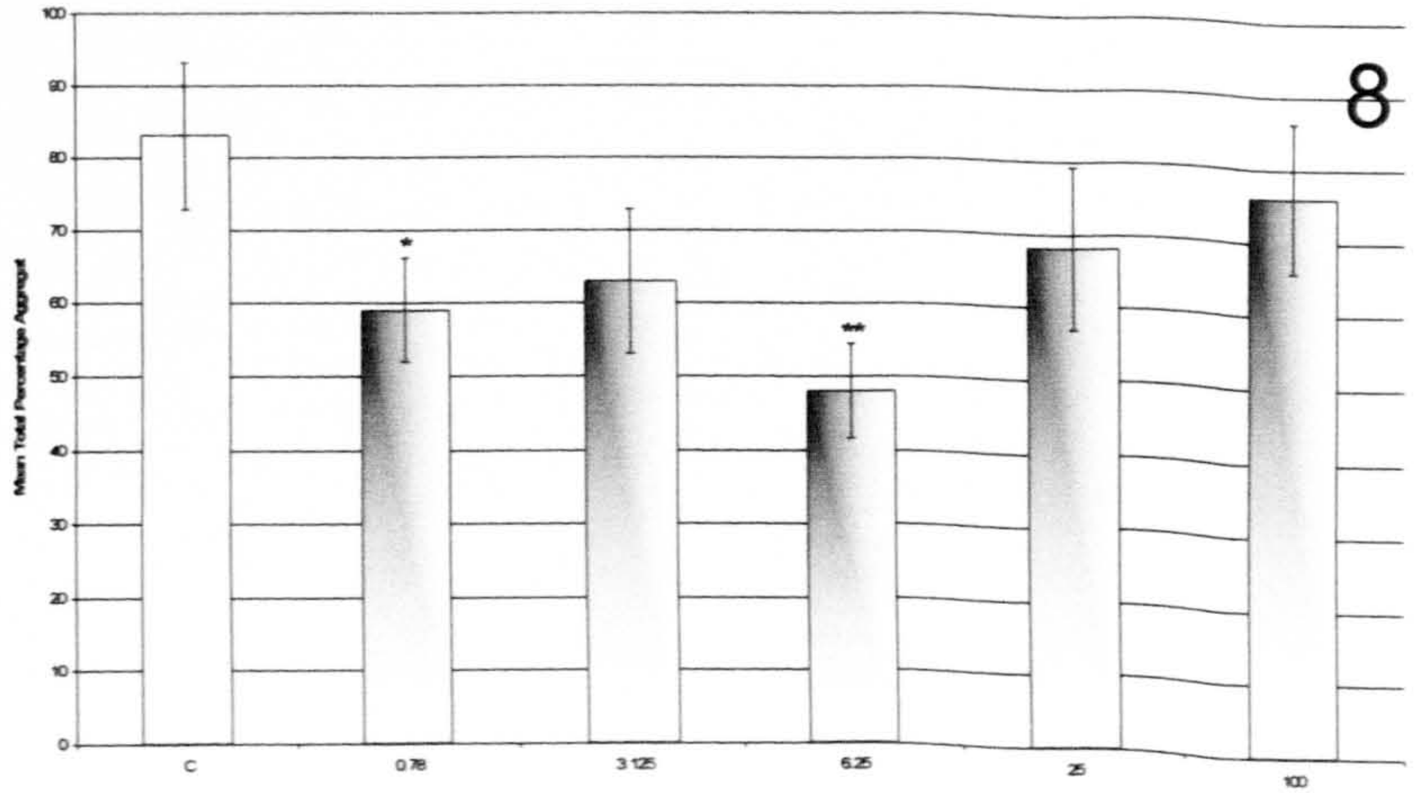


The effect of (1) cycloalliin; (2) ßC; (3) FA and (4) SAC at various concentrations on ADP-induced platelet aggregation *in vitro*. Aggregation curves were analysed for total percentage aggregation and values are expressed as means ± SE, n=6. *Statistically significant ($P<0.05$) when compared to the PBS control.









The effect of (5) SAMC; (6) SEC; (7) SMC and (8) SPC at various concentrations on ADP-induced platelet aggregation *in vitro*.Aggregation curves were analysed for total percentage aggregation and values are expressed as means ± SEM, n=6. *Statistically significant ($P<0.05$) when compared to the PBS control.

APPENDIX VI

Participant information sheet used for the clinical trial

PARTICIPANT INFORMATION SHEET

Mechanisms of inhibition of platelet aggregation by aged garlic extract (Kyolic)

Background information:

Platelet aggregation plays an important role in the development of cardiovascular disease and atherosclerosis. Circulating platelets adhere to fatty plaques within the blood vessel and, become activated. Once activated chemoattractant chemicals are released into the blood stream which recruit more platelets to the plaque site. Platelets then bind together to form a platelet clot which prevents blood flow and leads to the clinical presentation of disease ie. heart attack and stroke. Dietary supplementation with aged garlic extract (AGE) has been shown to be effective in the prevention of cardiovascular disease, as it reduces the aggregability of platelets, lowers blood cholesterol levels etc. However, the exact inhibitory nature is not clearly understood. This will be further investigated in this trial.

The study:

In vitro testing has shown that AGE inhibits agonist-induced platelet aggregation by reducing the ability of fibrinogen to bind to its receptor GPIIb/IIIa, increasing intracellular levels of cAMP and reducing the availability of calcium ions needed for aggregation to occur. The aim of this study is to measure these processes in vivo after dietary supplementation with AGE.

Participant role in study:

Both males and females; smokers and non-smokers are required for this clinical trial. You will give a blood sample (15-20 mls) at the beginning of the study. You will then take a specified daily dose (5 mls) of AGE for a period of two weeks. A blood sample will be taken again. You will stop taking the AGE for two weeks and a final blood sample will be taken. The aggregability of your platelets will be measured along with their ability to bind fibrinogen and intracellular cAMP concentrations.

You do not have to alter your lifestyle or diet and there is minimum pain involved in donating blood.

If at any time during the study, you wish to withdraw you can do so without prejudice.

Contact details:

Principle researcher: Miss Gillian Allison, 255 Max Perutz Building
G.L.Allison@2004.ljmu.ac.uk

Supervisors: Dr Khalid Rahman

Dr Gordon Lowe

APPENDIX VII

Participant questionnaire for clinical trial

QUESTIONNAIRE FOR CLINICAL TRIAL VOLUNTEERS

Name:

Sex (female/male)**:**

Age (years)**:**

Number of cigarettes smoked per day:

Average weekly alcohol consumption:
List type of alcohol consumed on a regular basis:

Known medical conditions (yes/no)**:**
If yes please list medical conditions:

Are you currently taking any prescribed medication (yes/no)**:**
If yes please list medications currently taking:

Do you take any dietary supplements (yes/no)**:**
If yes please list the type of supplements you take, ie. Multivitamins, cod liver oil capsules etc.

APPENDIX VIII

Individual data generated from clinical trial

Volunteer	**GP1A01**
Gender	Male
Age	34
No of cigarettes per day	10
Alcohol units/weeks	14
Medical conditions	NONE
Prescribed medication	NONE
Dietary supplements	Cod Liver Oil Capsules

%Aggregation	**ADP**	**AA**	**Adrenaline**	**Collagen**
Baseline	88	15	50	79
Following AGE	80	29	43	81
Washout	80	7	43	78

PAC1 binding	**Unactivated**	**ADP activated**	**CD42a expression**
Baseline	10.5	61.9	93.3
Following AGE	22.3	36.9	72.8
Washout	23.7	67.6	73.1

Intracellular cAMP levels	**Unactivated**	**ADP activated**
Baseline	0.61	3.6
Following AGE	1.2	1.8
Washout	0	1.8

Cholesterol analysis	mmol/l
Baseline	4.07
Following AGE	4.48
Washout	4.82

Volunteer	**GP1A02**
Gender	Male
Age	54
No of cigarettes per day	0
Alcohol units/weeks	0
Medical conditions	NONE
Prescribed medication	NONE
Dietary supplements	Cod Liver Oil Capsules

%Aggregation	**ADP**	**AA**	**Adrenaline**	**Collagen**
Baseline	79	39	67	91
Following AGE	60	0	24	60
Washout	87	65	67	17

PAC1 binding	**Unactivated**	**ADP activated**	**CD42a expression**
Baseline	12.8	75.9	88.6
Following AGE	26.9	41.3	72.5
Washout	25.3	70.6	46.3

Intracellular cAMP levels	**Unactivated**	**ADP activated**
Baseline	1.1	1
Following AGE	1.2	11
Washout	1.2	1.9

Cholesterol analysis	mmol/L
Baseline	3.96
Following AGE	3.76
Washout	3.96

Volunteer	**GP1A03**
Gender	Female
Age	22
No of cigarettes per day	0
Alcohol units/weeks	15
Medical conditions	NONE
Prescribed medication	NONE
Dietary supplements	NONE

%Aggregation	**ADP**	**AA**	**Adrenaline**	**Collagen**
Baseline	62	23	53	62
Following AGE	57	17	68	74
Washout	78	48	64	69

PAC1 binding (%)	**Unactivated**	**ADP activated**	**CD42a expression**
Baseline	17.5	74.8	93.1
Following AGE	28.4	52.3	79.1
Washout	30.7	56.5	56.7

Intracellular cAMP levels	**Unactivated**	**ADP activated**
Baseline	1.5	1.3
Following AGE	1.2	1.95
Washout	3.0	3.6

Cholesterol analysis	mmol/l
Baseline	3.78
Following AGE	3.50
Washout	3.86

Volunteer	**GP1A05**
Gender	Female
Age	44
No of cigarettes per day	0
Alcohol units/weeks	6
Medical conditions	NONE
Prescribed medication	NONE
Dietary supplements	B6, Zinc & Magnesium 1/day

%Aggregation	**ADP**	**AA**	**Adrenaline**	**Collagen**
Baseline	74	0	80	67
Following AGE	1	0	0	3
Washout	81	2	81	69

PAC1 binding	**Unactivated**	**ADP activated**	**CD42a expression**
Baseline	13.2	75.5	91
Following AGE	31.9	38.6	77.9
Washout	20.9	77.6	68.2

Intracellular cAMP levels	**Unactivated**	**ADP activated**
Baseline	1.05	1
Following AGE	1.9	1.8
Washout	1.0	1.0

Cholesterol analysis	mmol/L
Baseline	4.40
Following AGE	3.57
Washout	3.76

Volunteer	**GP1A06**
Gender	Female
Age	23
No of cigarettes per day	0
Alcohol units/weeks	0
Medical conditions	NONE
Prescribed medication	NONE
Dietary supplements	NONE

%Aggregation	**ADP**	**AA**	**Adrenaline**	**Collagen**
Baseline	36	51	66	90
Following AGE	67	0	53	74
Washout	56	59	62	91

PAC1 binding	**Unactivated**	**ADP activated**	**CD42a expression**
Baseline	11	31.8	90.1
Following AGE	33	75.9	76.5
Washout	31.3	42.0	77.8

Intracellular cAMP levels	**Unactivated**	**ADP activated**
Baseline	0.8	0.95
Following AGE	20	12
Washout	0.6	0.85

Cholesterol analysis	mmol/L
Baseline	3.29
Following AGE	3.19
Washout	2.80

Volunteer	**GP1B08**
Gender	Female
Age	26
No of cigarettes per day	0
Alcohol units/weeks	0
Medical conditions	NONE
Prescribed medication	NONE
Dietary supplements	NONE

%Aggregation	**ADP**	**AA**	**Adrenaline**	**Collagen**
Baseline	91	37	59	78
Following AGE	70	29	3	70
Washout	82	54	0	81

PAC1 binding	**Unactivated**	**ADP activated**	**CD42a expression**
Baseline	28.7	78.7	85.9
Following AGE	18.6	61.1	73.9
Washout	20.0	64.1	66.9

Intracellular cAMP levels	**Unactivated**	**ADP activated**
Baseline	0	1.3
Following AGE	2.1	1.7
Washout	0.35	1.6

Cholesterol analysis	mmol/L
Baseline	3.00
Following AGE	2.67
Washout	2.59

Volunteer	**GP1B09**
Gender	Male
Age	49
No of cigarettes per day	0
Alcohol units/weeks	5
Medical conditions	NONE
Prescribed medication	NONE
Dietary supplements	NONE

%Aggregation	**ADP**	**AA**	**Adrenaline**	**Collagen**
Baseline	80	19	38	82
Following AGE	56	8	7	67
Washout	71	0	56	90

PAC1 binding	**Unactivated**	**ADP activated**	**CD42a expression**
Baseline	35.9	86.7	78.3
Following AGE	26.5	58	69.9
Washout	22.3	70.7	67.8

Intracellular cAMP levels	**Unactivated**	**ADP activated**
Baseline	0	0
Following AGE	1.1	1.5
Washout	1.2	0.9

Cholesterol analysis	mmol/L
Baseline	4.25
Following AGE	4.12
Washout	4.04

Volunteer	GP1B10
Gender	Female
Age	48
No of cigarettes per day	0
Alcohol units/weeks	10
Medical conditions	Hypothyroid, depression
Prescribed medication	Thyroxine, venlafaxine
Dietary supplements	NONE

%Aggregation	ADP	AA	Adrenaline	Collagen
Baseline	72	62	45	86
Following AGE	64	0	7	52
Washout	104	0	95	103

PAC1 binding	Unactivated	ADP activated	CD42a expression
Baseline	22.3	77.2	84.1
Following AGE	21.7	70.9	73.1
Washout	19.9	39.1	86.0

Intracellular cAMP levels	Unactivated	ADP activated
Baseline	0	0.12
Following AGE	0.91	0.75
Washout	0.61	0.61

Cholesterol analysis	mmol/L
Baseline	4.35
Following AGE	5.26
Washout	5.31

Volunteer	**GP2A12**
Gender	Female
Age	62
No of cigarettes per day	0
Alcohol units/weeks	10
Medical conditions	NONE
Prescribed medication	NONE
Dietary supplements	NONE

%Aggregation	**ADP**	**AA**	**Adrenaline**	**Collagen**
Baseline	74	10	74	89
Following AGE	66	12	12	80
Washout	75	7	35	70

PAC1 binding	**Unactivated**	**ADP activated**	**CD42a expression**
Baseline	12.6	33.9	78.9
Following AGE	26.1	68.7	76.9
Washout	26.3	53.9	60.8

Intracellular cAMP levels	**Unactivated**	**ADP activated**
Baseline	0	0.95
Following AGE	1.1	4
Washout	1.8	1.6

Cholesterol analysis	mmol/L
Baseline	4.30
Following AGE	4.87
Washout	4.58

Volunteer	**GP2A13**
Gender	Female
Age	47
No of cigarettes per day	0
Alcohol units/weeks	5
Medical conditions	Meniere's
Prescribed medication	Betahistine
Dietary supplements	NONE

%Aggregation	**ADP**	**AA**	**Adrenaline**	**Collagen**
Baseline	88	12	20	89
Following AGE	71	9	9	65
Washout	77	4	73	69

PAC1 binding	**Unactivated**	**ADP activated**	**CD42a expression**
Baseline	12.1	28.6	82.4
Following AGE	22.3	75.5	78.6
Washout	36.8	68.6	72.8

Intracellular cAMP levels	**Unactivated**	**ADP activated**
Baseline	0	1.8
Following AGE	1.2	6
Washout	0.95	0.95

Cholesterol analysis	mmol/L
Baseline	5.62
Following AGE	4.82
Washout	4.14

Volunteer	**GP2A17**
Gender	Male
Age	46
No of cigarettes per day	0
Alcohol units/weeks	14
Medical conditions	Hypertension, IgA nethropathy
Prescribed medication	Nifedipine, Irbestartin, Enapril
Dietary supplements	

%Aggregation	**ADP**	**AA**	**Adrenaline**	**Collagen**
Baseline	96	0	87	90
Following AGE	88	26	78	84
Washout	78	0	62	70

PAC1 binding	**Unactivated**	**ADP activated**	**CD42a expression**
Baseline	17.9	67.9	89.8
Following AGE	21.1	85.1	66.3
Washout	35.5	72.8	70.0

Intracellular cAMP levels	**Unactivated**	**ADP activated**
Baseline	0.75	1.4
Following AGE	8	1.95
Washout	1.9	1.1

Cholesterol analysis	mmol/L
Baseline	5.18
Following AGE	5.00
Washout	5.31

Volunteer	**GP2A21**
Gender	Female
Age	29
No of cigarettes per day	4
Alcohol units/weeks	19
Medical conditions	NONE
Prescribed medication	NONE
Dietary supplements	Omega 3-6-9, 1000mg/day

%Aggregation	**ADP**	**AA**	**Adrenaline**	**Collagen**
Baseline	60	1	2	72
Following AGE	45	4	9	46
Washout	53	0	17	77

PAC1 binding	**Unactivated**	**ADP activated**	**CD42a expression**
Baseline	13.4	45.9	88.7
Following AGE	18.5	78.4	78.2
Washout	18.1	73.7	88.6

Intracellular cAMP levels	**Unactivated**	**ADP activated**
Baseline	1	1.1
Following AGE	1.1	1.6
Washout	0.75	0.95

Cholesterol analysis	mmol/L
Baseline	4.84
Following AGE	4.14
Washout	2.72

Volunteer	**GP2B18**
Gender	Female
Age	27
No of cigarettes per day	18
Alcohol units/weeks	0
Medical conditions	NONE
Prescribed medication	Contraceptive pill
Dietary supplements	NONE

%Aggregation	**ADP**	**AA**	**Adrenaline**	**Collagen**
Baseline	57	0	52	84
Following AGE	51	15	20	0
Washout	98	41	72	93

PAC1 binding	**Unactivated**	**ADP activated**	**CD42a expression**
Baseline	6.1	62.5	90.3
Following AGE	20.2	70.9	61.3
Washout	27.2	54.0	81.8

Intracellular cAMP levels	**Unactivated**	**ADP activated**
Baseline	0	1.05
Following AGE	11	18
Washout	2.5	1.6

Cholesterol analysis	mmol/L
Baseline	5.31
Following AGE	4.75
Washout	3.99

Volunteer	**GP2B19**
Gender	Male
Age	60
No of cigarettes per day	0
Alcohol units/weeks	14
Medical conditions	Mild hypercholestemia
Prescribed medication	NONE
Dietary supplements	NONE

%Aggregation	**ADP**	**AA**	**Adrenaline**	**Collagen**
Baseline	88	31	70	57
Following AGE	80	14	43	58
Washout	65	6	49	65

PAC1 binding	**Unactivated**	**ADP activated**	**CD42a expression**
Baseline	14.2	31.9	77.9
Following AGE	29.3	70.8	55.3
Washout	20.4	47.4	75.8

Intracellular cAMP levels	**Unactivated**	**ADP activated**
Baseline	1.5	1.6
Following AGE	1.7	6
Washout	1.3	1.8

Cholesterol analysis	mmol/L
Baseline	4.38
Following AGE	4.04
Washout	3.99

Volunteer	**GP2A20**
Gender	Female
Age	41
No of cigarettes per day	0
Alcohol units/weeks	2
Medical conditions	NONE
Prescribed medication	NONE
Dietary supplements	NONE

%Aggregation	**ADP**	**AA**	**Adrenaline**	**Collagen**
Baseline	69	17	70	83
Following AGE	63	0	0	98
Washout	82	0	82	73

PAC1 binding	**Unactivated**	**ADP activated**	**CD42a expression**
Baseline	34.3	87.8	85.7
Following AGE	29.1	66.1	68.7
Washout	17.4	51.4	76.8

Intracellular cAMP levels	**Unactivated**	**ADP activated**
Baseline	0	0
Following AGE	1.1	0.65
Washout	0	0

Cholesterol analysis	mmol/L
Baseline	3.83
Following AGE	3.99
Washout	3.96

Volunteer	**GP1B07**
Gender	Male
Age	28
No of cigarettes per day	0
Alcohol units/weeks	12
Medical conditions	NONE
Prescribed medication	NONE
Dietary supplements	NONE

%Aggregation	**ADP**	**AA**	**Adrenaline**	**Collagen**
Baseline	79	0	85	37
Following AGE	67	0	78	3
Washout	103	96	108	106

PAC1 binding	**Unactivated**	**ADP activated**	**CD42a expression**
Baseline	13.3	63	89.4
Following AGE	28.8	83.4	79.7
Washout	25.9	46.6	79.4

Intracellular cAMP levels	**Unactivated**	**ADP activated**
Baseline	1.2	1.6
Following AGE	1.8	1.95
Washout	0.85	1.3

Cholesterol analysis	mmol/L
Baseline	3.24
Following AGE	2.80
Washout	3.32

Volunteer	**GP1B22**
Gender	Female
Age	57
No of cigarettes per day	0
Alcohol units/weeks	14
Medical conditions	NONE
Prescribed medication	NONE
Dietary supplements	Cod liver oil multivitamins

%Aggregation	**ADP**	**AA**	**Adrenaline**	**Collagen**
Baseline	92	4	78	79
Following AGE	80	0	64	69
Washout	91	0	73	69

PAC1 binding	**Inactivated**	**ADP activated**	**CD42a expression**
Baseline	25.7	74.9	77.0
Following AGE	20.5	59.0	82.1
Washout	26.1	68.3	71.0

Intracellular cAMP levels	**Inactivated**	**ADP activated**
Baseline	0.85	1.3
Following AGE	4.5	5
Washout	0	0

Cholesterol analysis	mmol/L
Baseline	5.62
Following AGE	5.21
Washout	5.60