Investigations into effects of 12-lipoxygenase and NADPH oxidase on platelet activity, and influences of dietary dark chocolate

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Declaration

I hereby declare that all the work presented in this thesis is my own.

Abstract

Platelets play a pivotal role in both normal hemostasis and pathological bleeding and importantly also contribute to the development of atherothrombosis. Even though platelet function tests traditionally are utilised mainly for the diagnosis and management of patients presenting with bleeding problems rather than thrombosis, new and improved platelet function tests are now increasingly used to monitor anti-platelet therapy in patients and to identify patients at risk of arterial disease. Based on light transmission traditional aggregometry, this thesis reports data from a new model of platelet aggregation using a modified 96-well plate format. This method allows examination of many agonists at a range of concentrations at the same time. Thus, more information can be collated about different aspects of platelet function and smaller assay volumes can be used while still obtaining reliable results. To further utilise this method, agonist combinations were used in the 96-well plate approach that resemble the actions of machines such as the PFA-100, which uses combined agonists within a cartridge, but at much lower cost. Platelet cyclooxygenase has been widely studied; however, the functions of platelet 12-lipoxygenase and NADPH oxidase in platelets are still generally not understood. Data presented here demonstrate that both pathways are partly essential in platelet activation following exposure to stimulatory agonists. To further explore the relationship between dietary intake and the risk of atherothrombosis, an *in vivo* study was performed to observe the antiplatelet effects following from consumption of dark chocolate in baseline hypertensive patients. Based on findings in this thesis, it can be

concluded that this new method of evaluating platelet aggregation and adhesion in a 96-well plate format is very useful, and that new observations into influences on platelets of pathways other than cyclooxygenase may be beneficial in the development of new antiplatelet drugs.

Publication

Additive Effects of Collagen and Adrenaline on Platelet Aggregation in 96-well Plates
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Inhibition by theobromine, a cocoa methylxanthine, of platelet aggregation and adhesion stimulated by various agonists

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Influence of endothelial cells on platelet aggregation and adhesion in 96-well plates

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Linear relationship between platelet aggregation and thromboxane a_2 production

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Linear relationship between collagen-induced platelet aggregation and thromboxane A_2 production

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Evaluation of Aspirin Inhibition on Platelet Induced By Combined Collagen and Adrenaline in 96-Well Plate

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Abbreviations

12(S)-HETE	12(S)-hydroxyeicosatetranoic acid
12-LOX	12-lipoxygenase
12(S)-HPETE	12(S)-hydroperoxyeicosatetranoic acid
AA	Arachidonic acid
ACE	angiotension converting enzyme
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CADP	Collagen/ADP
cAMP	Cyclic AMP
CDC	cinnamyl-3, 4-dihydroxy-α-cyanocinnamade
CEPI	Collagen/epinephrine
cGMP	Cyclic GMP
CLASS	Celecoxib Long-term Arthritis Safety Study
COX	Cyclooxygenase
CT	Closure time
DPI	Diphenylene iodonium
GP	glycoprotein
GPCR	G-protein couple receptor
LTA	Light transmission aggregometry
MDA	malonylaldehyde
NO	nitric oxide

NSAIDs	Non-steroidal anti-inflammatory drugs
PAR	Protease-activated receptor
PBS	Phosphate buffer saline
PFA-100	Platelet function analyzer-100
PGI_2	Prostacyclin
PLA_2	Phoslipase A_2
PLC	Phospholipase C
PPP	Platelet poor plasma
PRP	Platelet-rich plasma
ROS	Reactive oxygen species
SNP	Sodium nitroprusside
T2DM	Type-II Diabetes Mellitus
TXA_2	Thromboxane A_2
TxB_2	Thromboxane B_2
VIGOR	The Vioxx Gastrointestinal Outcomes Research
vWF	von Willebrand Factor

CHAPTER ONE:

INTRODUCTION

1.1 PLATELET

1.1.1 Introduction

Platelets are derived and released from bone marrow megakaryocytes in the bone marrow. Once the polyploid cell reaches maturation, it can form proplatelet extensions from which platelets are released (Patel *et al.*, 2005). Platelets are the smallest formed elements in the blood, and circulate as anucleate discoid cells with a mean volume of about 7 to 9 fL and have a life span of approximately 8-10 days. Thrombopoietin is the major hormonal regulator of platelet production that binds to c-mpl, a specific receptor expressed on megakaryocytes and platelets. However, recently nitric oxide (NO) has also been reported to stimulate platelet production from megakaryocytes (Battinelli *et al.*, 2001).

Platelets consist of many organelles, such as mitochondria, lysosomes and three types of granules. Dense granules release adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin and calcium ions. Alpha granules release constituents include fibrinogen, growth factors (platelet derived growth factor, β-transforming growth factor), and cytokines (platelet factor 4, neutrofil-activating peptide-2, β-thromboglobulin). Lysosomal granules contain acid proteases, acid glycosidases, acid phosphatases, and aryl sulphatases (McNicol *et al.*, 2003). Plasma membrane of the platelets consists of

glycoproteins (GPs) that bind specific adhesive proteins to promote platelet-to-surface interactions (adhesion) and platelet-to-platelet interactions (aggregation) (Rand *et al.*, 2005). Recent studies demonstrate that platelets and many of their products are important not only in hemostasis, but also essential in immunoregulation and inflammation as platelets produce mediators that regulate inflammation.

Normal endothelium not only acts as physical barrier, but also maintains local vascular homeostasis by assuring regular vasopermeability, by promoting vasodilatation, by limiting activation of coagulation cascade and by inhibiting platelet aggregation, white blood cell adhesion and smooth muscle cell proliferation (Zardi *et al.*, 2005). Under normal conditions of blood flow and shear stress, the vascular source of nitric oxide (NO) which is synthesized from *L*-arginine, is likely derived from biochemical agonist- and shear-dependent release of endothelial NO to prevent excessive platelet activation (Cooke *et al.*, 1990). Endothelial NO attenuate intracellular signalling of platelet activation by increasing levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (Brass, 2003). There are evidence showing that in suspended platelets, NO inhibits platelet activation by targeting inositol triphoshate (IP_3) receptor (Cavallini *et al.*, 1996), TXA_2 receptor (Reid *et al.*, 2003) and vasodilator-stimulated phosphoprotein (VASP) (Halbrugge *et al.*, 1990). Interestingly, NO also causes down-regulation of Ca^{2+} levels thus preventing platelet granule secretion and platelet GPIIb-IIIa activation (Le Quan Sang *et al.*, 1996). NO also inhibits platelet activation independent of cGMP pathway by targeting Ca^{2+} and PI3kinase thus inhibit GPIIb-IIIa-mediated adhesion (Oberprieler *et al.*, 2007).

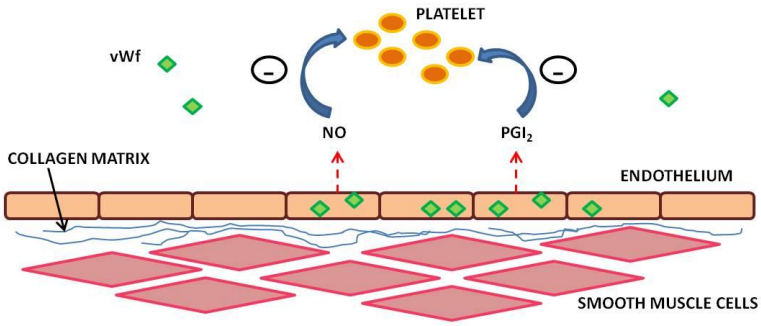
Under conditions of endothelial dysfunction, generation of platelet NO from may become important in regulating platelet responses with active constitutive NO synthase has been found in platelets (Sase *et al.*, 1995). A previous important study showed that NO synthase inhibitor, L-N-nitroarginine methyl ester (L-NAME) reduce NO production *in vitro* by causing a decrease of cGMP level and increase of serotonin release (Freedman *et al.*, 1996). In addition, this study also suggested that platelet-derived NO regulates platelet recruitment to the growing thrombus. Endothelium expresses constitutive form of nitric oxide synthase (NOS), endothelial NOS (eNOS) and under certain condition such as inflammation, could also express inducible NOS (iNOS) (Naseem, 2005). Recently, platelets also have been shown to express NOS and produce NO to inhibit other platelet recruitment thus keeping aggregation under control (Pronai *et al.*, 1991) (Vasta *et al.*, 1995) (Sase *et al.*, 1995). Sase *et al.* (1995) demonstrated that only eNOS is present in platelet but not iNOS or nNOS, as shown by PCR amplification of platelet cDNA by eNOS primers but not iNOS or nNOS primers.

Type of NOS	Location	Function
Endothelial NOS (eNOS)	-plasma membrane region of endothelial cells	-vasodilation -inhibits platelet aggregation
Inducible NOS (iNOS)	- induced by immunostimulatory cytokines, bacterial products or infection in a number of cells, including endothelium, hepatocytes, monocytes, mast cells, macrophages and smooth muscle cells	-involves in inflammation as immune defense
Neuronal NOS (nNOS)	-neuron, synaptic spines -skeletal muscle -cardiac muscle -smooth muscle	-control of blood flow -control of muscle contractility

Table 1: *Nitric oxide synthase (NOS) isoforms, localisations and functions.*

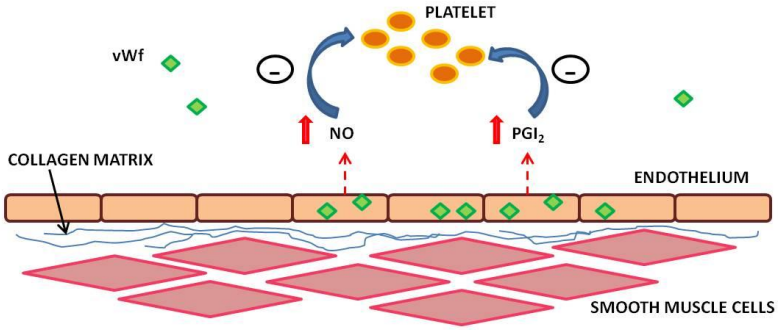
Apart from NO, prostacyclin (PGI_2), another major product of arachidonic acid metabolism is also synthesized in platelets (Hammarstrom *et al.*, 1977) and vascular endothelium (Marcus *et al.*, 1978), respectively. It has been demonstrated that an increase in shear stress was shown to increase PGI_2 synthesis by endothelium (Frangos *et al.*, 1985) and upregulate mRNA levels for COX-1, COX-2, and prostacyclin synthase (PGIS) *in vitro* (Okahara *et al.*, 1998). An earlier study showed that PGI_2 inhibits platelet aggregation and thrombus formation *in vivo* (Higgs *et al.*, 1978). PGI_2, synthesized from the conversion of PGH_2 to PGI_2 by PGIS binds to IP receptors on platelets (Vane *et al.*, 1998) and has potent vasodilator and anti-thrombotic activities (Vane, 1971). PGI_2 and IP mimetics such as iloprost and cicaprost inhibits platelet aggregation by activating adenylyl cyclase leads to an increase of cAMP and reduce platelet intracellular Ca^{2+} level (Roma A, 1996).

a)



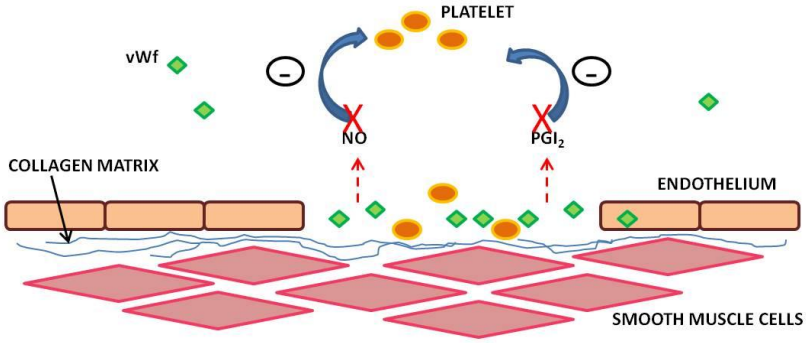
Normal shear stress

b)



High shear stress

c)



Endothelial dysfunction

Figure 1.1: Endothelium produces nitric oxide (NO) and prostacyclin (PGI_2) that acts as vasodilator and platelet inhibitor (a). (b) NO and PGI_2 production are increase during elevated sheer stress. (c) NO and PGI_2 bioavailability is decrease in endothelial dysfunction.

Platelets circulate in the blood stream without adhering to the blood vessel endothelium. However, in response to endothelial disruption, platelets adhere within seconds to subendothelial matrix molecules, in particular to collagen. Activated platelets immediately change shape, losing their discoid shape, and form tiny spheres with numerous projecting pseudopods (Yardumian *et al.*, 1986). Subsequently, additional platelets and leukocytes (neutrophils, monocytes) are recruited to the initial platelet layer, resulting in the formation of a thrombus capable of provisionally occluding the vessel perforation. Hemostatic plug stabilised by fibrin is formed at the site of vessel injury when a blood vessel is injured. However, platelet-fibrin thrombi forms on ruptured atherosclerotic plaques are responsible for the clinical complications of atherosclerosis, a process of plaque formation in the lining of the arteries resulting different development of diseases based on which arteries are affected such as peripheral or coronary.

1.1.2 Platelet Adhesion

Platelet adhesion to extracellular matrix is a complex event involving binding of several membrane glycoproteins to plasma and exposed subendothelial tissue components such as collagen, fibrinogen and von Willebrand factor (vWF) to form a fragile monolayer. Human possess at least 25 forms of collagen (Hashimoto *et al.*, 2002) and many of these (I,III,IV,V,IV,VIII,XII,XIII, and XIV) are present in the blood vessel wall (Barnes *et al.*, 1999). In addition, type IV collagen is present in the subendothelial basement membrane. vWF is an adhesive protein that is secreted by endothelial cells and platelets, and is present in the

subendothelium and in plasma. Platelet adhesion is mediated by several cell-surface receptors on the platelet such as α2β1 intergrin and glycoprotein (GP) VI collagen receptors and GPIb, the von Willebrand Factor receptor (McNicol *et al.*, 2003). Spreading of platelet adhesion is accompanied by the secretion or synthesis of several prothrombotic factors such as adenosine 5'-diphosphate (ADP), serotonin and thromboxane A_2 which act in an autocrine/paracrine fashion and activate or prime approaching platelets.

1.1.3 Platelet Activation

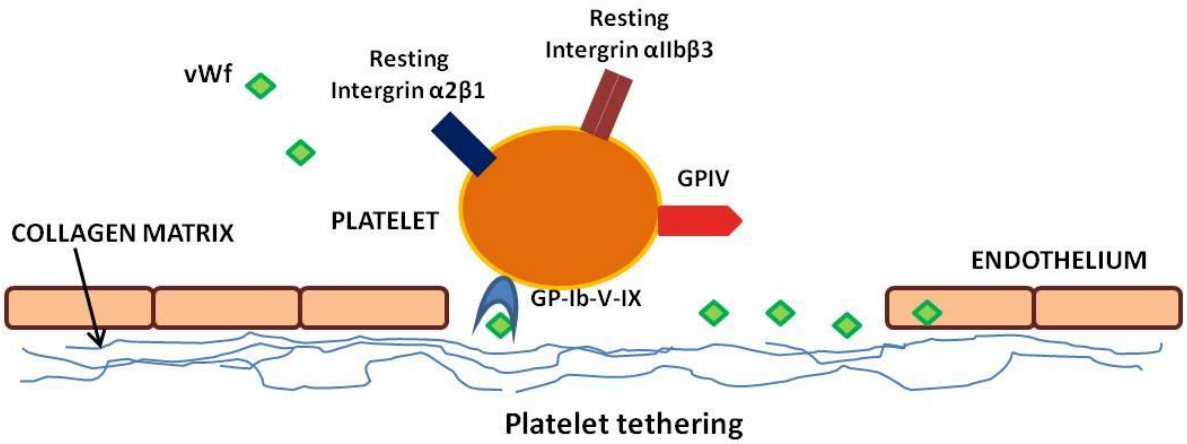
Platelet adhesion is followed by platelet secretion and activation. However, platelets may have been activated prior to adhesion by exposure to circulating mediators. Platelets are activated by several agents that play a role in recruiting additional platelets to the site of injury which lead to the formation of haemostatic plug or aggregate. Recruitment of other platelets crucially depends on amplification systems provided by autocrine and paracrine factors such as ADP and thromboxane A_2. Platelet adhesion to the subendothelium stimulates the secretion of platelet dense and alpha storage granule contents, including ADP from the dense granules, and formation of thromboxane A_2 (TxA_2), both of which promote aggregation. Activated platelets also enhance coagulation by providing phosphatidylserine on the membrane surface on which the coagulation factor complexes can assemble, thus provide fibrin for the stabilisation of the newly formed thrombus. The

generation of thrombin by the prothrombinase complex mediated predominantly on the surface of the activated platelets further stimulates platelets.

1.1.4 Platelet Aggregation

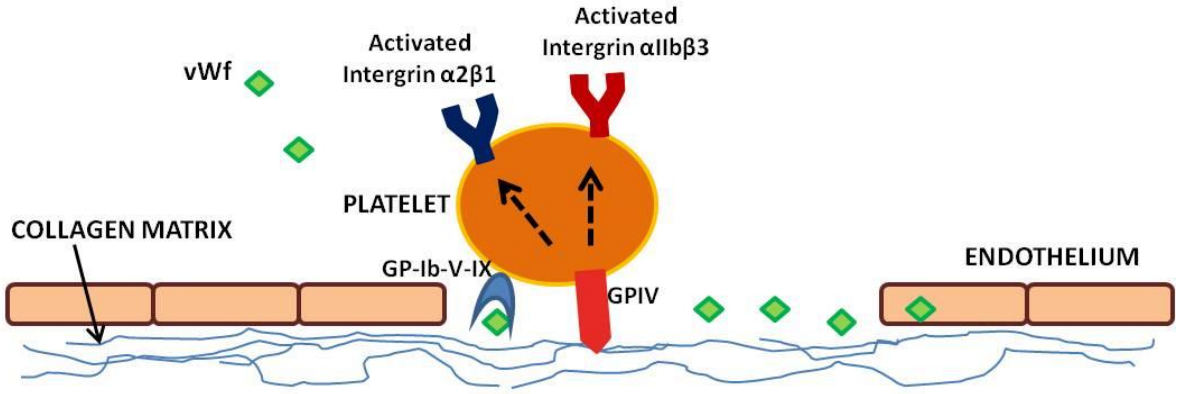
Platelet aggregation at site of vascular injury is an important event for the formation of the hemostatic plug and also for the development of thrombi at site of atherosclerotic plaque rupture. Platelet aggregation occurs when other platelets in free-flowing blood subsequently adhere to the initial layer of adherent platelets (Kulkarni *et al.*, 2000). Intergrin $\alpha_{IIb}\beta_3$ plays an exclusive role in mediating platelet-platelet adhesion contacts in which its activation change their conformation from a low- to a high-affinity receptor capable of binding soluble fibrinogen. In addition, the dimeric form of fibrinogen enables it to cross-link adjacent activated platelets leading to stable platelet aggregation (Kulkarni *et al.*, 2000).

a)



Platelet tethering

b)



Platelet activation

c)

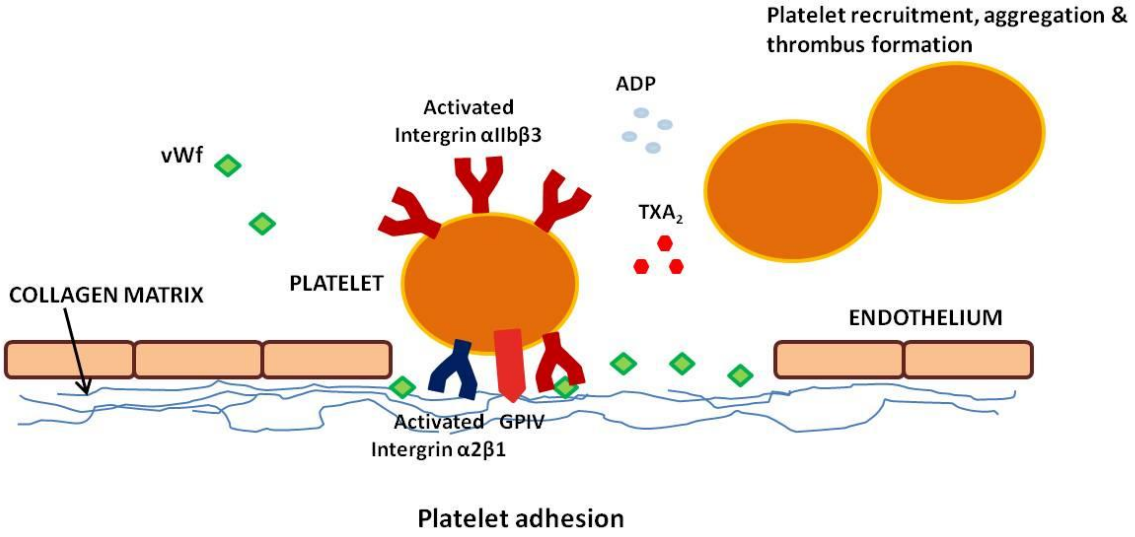


Figure 1.2: *Intergrin activation during platelet activation by exposed collagen matrix.* Platelet tethering occurs when von Willebrand factor (vWf) on endothelium binds to GPIb-V-IX (a), followed by the binding to exposed collagen matrix with platelet GPIV receptors to activate platelets (b). This will activates intergrin α2β1 and intergrin αIIbβ3 that will binds to collagen and vWf, respectively to stimulate platelet adhesion to site of injuries (c). Platelet activation also causes granule secretion of secondary agonists such as TXA_2 and ADP, thus promotes platelet recruitment and platelet aggregation.

1.1.5 Platelet Agonists

In the process of maintaining hemostasis, primary platelet plug will be formed as result of platelet activation to stop bleeding and prevent blood loss. Platelet activator can be divided into primary agonist or secondary agonist. Primary agonist includes von Willebrand factor which binds to GPIb-IX receptor in the platelet membrane, and collagen that binds to GPIb and GPIIb-IIIa in high shear stress condition or GPVI and GPIa/IIa in low shear (Farndale, 2006). Platelet activation by vWf and collagen leads to platelet adhesion at site of endothelial injury and stimulates platelet granule release of secondary agonist. Secondary agonist includes ADP, TXA_2, serotonin and PAF which further activates platelet recruitment and aggregation. Platelet activation will also cause conformational changes to GPIIb-IIIa that provide high affinity binding site for soluble fibrinogen. This is important to strengthen the primary platelet plug by fibrinogen that acts as bridging molecule between GPIIb-IIIa in adjacent activated platelets (Kulkarni *et al.*, 2000).

1.1.5.1 Collagen

As a consequence of damage to blood vessels, subendothelium is exposed to the bloodstream, which results in circulating platelets reacting with the collagen in the subendothelium, adhering to it and forming aggregates on the damaged surface. Collagen is a very important physiological activator because of its high content in the subendothelium and its strong ability to induce platelet aggregation and adhesion (Jung *et*

al., 2000). Platelets adhere to exposed collagen fibers and undego activation via a tyrosine kinase-dependent signalling pathway (Gibbins *et al.*, 1998), resulting in the activation of intergrins (adhesive proteins), which in turn leads to platelet adhesion and aggregation. Collagen binds directly to several receptors on the platelet surface, notably intergrin $\alpha_2\beta_1$ and glycoprotein VI (GPVI), which play a major role in cell adhesion and activation (Jung *et al.*, 2000). A previous study showed that in a patient who demonstrated no reactivity to collagen, the normal level of intergrin $\alpha_2\beta_1$ was reduced by about 80-85% (Nieuwenhuis *et al.*, 1986). Activation causes an increase in the binding capability of the fibrinogen receptor, intergrin $\alpha_{IIb}\beta_3$ and the secretion of various mediators that culminate in the formation of an irreversible platelet aggregate, or hemostatic plug. Even though the precise function of intergrin $\alpha_2\beta_1$ and GPVI is still a matter of debate; $\alpha_2\beta_1$ intergrin appears to be mainly involved in platelet adhesion (Jung *et al.*, 2000), while the GPVI is responsible for activation through a signal tranduction pathway involving FcRγ chain, tyrosine phosphorylation and phospholipase C (PLC) γ2 activation (Nieswandt *et al.*, 2003).

1.1.5.2 Thrombin

Thrombin is a strong agonist that can cause complete secretion of the contents of both the α-granules and the dense granules, regardless of the concentration of Ca^{2+} in the suspending medium, and independent of strirring, aggregation, or the presence of an inhibitor such as aspirin (Rand *et al.*, 1996). It is known that thrombin-mediated platelet aggregation is critical for acute vascular thrombosis following mechanical injury or rupture

of artherosclerotic plaques. Thrombin is generated from the conversion of prothrombin by Factor Xa in the coagulation pathway which its main role is to convert fibrinogen to fibrin (Monroe *et al.*, 2002). There are at least three receptors for thrombin on human platelets, two protease-activated receptors (PAR), PAR1 and PAR4, and also GPIbα. PAR-1 is a high-affinity receptor for platelet activation at low concentrations of thrombin, whereas PAR4 is a low-affinity receptor that mediates thrombin signalling at high concentrations. Thrombin cleaves the receptors within the large N-terminal extracellular domain, creating a new amino terminal, SFLLRN and GYPGQV respectively. Therefore, specific agonist peptides that resemble the new N-terminus, have been designed, for PAR1 the most often used is SFLLRN (PAR1-AP), whilst for PAR4 the most potent is AYPGKF (PAR4-AP) (Ramström *et al.*, 2008). TRAP-6, a synthetic peptide of a sequence SFLLRN-NH2 can fully stimulate PAR-1 receptor function although the potency is 1000-fold less than that of native thrombin (Derian *et al.*, 2003). However, the availability of synthetic peptides for PAR receptor has provided the means to understand interaction of ligand-receptor interaction and to fascilitate drug design.

1.1.5.3 Ristocetin

Another platelet agonist is the antibiotic ristocetin, that is the presence of normal platelets and a normal complement of von Willebrand factor (vWf) antigen causes GPIb/vWF-dependent platelet agglutination. However, aggregation induced by ristocetin at concentrations of up to 1.2mg/mL, may change at concentrations above 1.5mg/mL to

platelet clumping due to activation with fibrinogen (Yardumian *et al.*, 1986 145). In platelet-rich- plasma (PRP), ristocetin causes a primary and secondary wave of aggregation due to granule release. vWf plays an essential role in platelet aggregation. Therefore the quantitative determination of its levels in patients with von Willebrand disease is very important. The concentration of vWf in plasma can be assessed by its ability to promote agglutination of platelets in the presence of the antibiotic ristocetin (Ermens *et al.*, 1995). The ristocetin cofactor (vWf:RCof) assay that measures vWF activity in plasma involves the mixing of patient's plasma and commercial formalin-fixed or fresh washed platelets with a standard amount of ristocetin, and then determination of the velocity of platelet agglutination by the use of aggregometry (Ewenstein, 2001).

1.1.5.4 Thromboxane A_2

Arachidonic acid (AA) is an essential fatty acid precursor in the biosynthesis of leukotrienes, prostaglandins, and thromboxanes. In platelets, cyclooxygenase (COX)-1 converts AA to thromboxane A_2 (TXA_2), which induces and mediates aggregation, and as such is an important pathway in platelet aggregation. In addition, exposure of platelets to AA not only results in aggregation but also secretion of the dense- and α-granule contents dependent upon the conversion of AA into TXA_2 (Linder *et al.*, 1979). It is interesting to note that despite the fact that AA induces platelet activation, high exogenous AA also attenuated platelet activation by increases cAMP levels and decreases cytoplasmic Ca^{2+} concentration, thus inhibiting platelet aggregation and secretion (Kowalska *et al.*, 1988).

Agonist-induced platelet stimulation produces a rise in the cytoplasmic Ca^{2+} concentration caused by both release of Ca^{2+} from the intracellular stores and entry through plasma membrane that leads to platelet activation. Previous studies have shown that AA stimulates platelets by increasing Ca^{2+} concentration which was inhibited by COX inhibitors but imitated by the TXB_2 mimetic, U46619 (Alonso *et al.*, 1990). The measurement of TXB_2 levels can be employed to quantify the inhibition of platelet COX-1 in AA-induced *ex vivo* platelet aggregation. In addition to that, AA used as an agonist in *ex vivo* aggregometry is specific for COX-1 mediated aggregation (Burke *et al.*, 2003).

The receptor for TXA_2 belongs to the group of G-protein-coupled receptors (Offermanns *et al.*, 1994). Initial studies showed that G_{12} and G_{13} are important in transmembrane signal transduction upon stimulation by TXA_2 and thrombin, indicating that both GPCR receptors are involved in platelet activation pathway (Offermanns *et al.*, 1994). Signal tranduction by $G_{12/13}$-coupled TP receptors causes platelet shape change, and in concert with G_i-mediated signalling fully activates platelet aggregation and degranulation (Klages *et al.*, 1999). This observation was followed by another finding that showed TXA_2 binds to the specific TPα and TPβ receptor subtypes leading to G-protein-coupled receptor, G_q and $G_{12/13}$ signalling (Dorsam *et al.*, 2002). U46619, the thromboxane analogue triggers some platelet responses, for example platelet shape change and adhesion to surface bound fibrinogen. However, there is no detectable aggregation or secretion at low doses of U46619 that were effective at stimulating tyrosine phosphorylation (Minuz *et al.*, 2006).

1.1.5.5 ADP

As a secondary agonist, ADP is a weak aggregating agent and plays a role in propagation of platelet activation that is greatly influenced by extracellular conditions such as Ca^{2+} levels or the presence of platelet inhibitors especially aspirin. The common concentrations of ADP used in assessment of platelet aggregation are 1 to 10μM with lower concentrations (1-3μM) producing either single aggregation response curves or clearly biphasic curves. ADP was identified as a factor derived from erythrocytes which influenced platelet adhesion to glass and induced platelet aggregation (Gachet *et al.*, 2006) and plays a very important physiological role because it is one of the positive feedback mechanisms that act to spread and enhance platelet aggregation for hemostasis. Together with other aggregating agents, ADP causes an increase in intracellular calcium from internal sequestered stores, as well as extracellularly by influx via calcium channels. Apart from increasing intracellular Ca^{2+} levels, ADP also inhibits stimulation of adenylyl cyclase (Cusack *et al.*, 2000). As adenylate cyclase stimulation inhibits platelet activation, it seems likely that inhibition of stimulated adenylyl cyclase by ADP has some significance as it may offset effects of stimulators of adenylyl cyclase, such as adenosine and prostacyclin, to which platelets may be exposed *in vivo*. ADP-induced platelet aggregation results from the co-activation of two P2 receptors, G-protein-coupled receptors (GPCRs), $P2Y_1$ and $P2Y_{12}$ (Mangin *et al.*, 2004). The $P2Y_1$ receptor is responsible for the calcium mobilisation by ADP receptor through the activation of phospholipase (Jin *et al.*, 1998). In addition, the $P2Y_1$ receptor is responsible for inositol triphosphate formation through activation of

phosholipase C, leading to mobilisation of calcium, platelet shape change and transient aggregation in response to ADP (Kunapuli *et al.*, 2003). The $P2Y_{12}$ receptor is the target for antithrombotic thienopyridine compounds such as clopidogrel and is responsible for completion and amplification of the response to ADP itself, and to other agonists (Gachet, 2001).

1.1.5.6 Adrenaline

Adrenaline is well known as an agonist that causes platelet aggregation in citrated platelet rich plasma (Nakamura *et al.*, 1997) detected either by an increase in light transmission or by a decrease in the single platelet content of the suspension (Shattil *et al.*, 1989). Adrenaline binds to α_2-adrenergic receptors and stimulates variable platelet aggregation and secretion. However, previous studies have shown that adrenaline does not function as a single platelet agonist but, rather, that it enhances activation initiated by other agonists (Dunlop *et al.*, 2000). In addition, adrenaline does not induce platelet shape change leading to aggregation, at the common final concentrations used of around 1-10µM (Yardumian *et al.*, 1986). Adrenaline increases thromboxane A_2 (TxA_2) formation by promoting low-level activation of phospholipase A_2 that releases free arachidonic acid which is converted by cyclooxygenase to TxA_2 (Banga *et al.*, 1986). In contrast, adrenaline is the least consistent agonist in *ex vivo* tests. In particular, if a subject has taken aspirin or any other drugs that inhibit TxA_2 formation the platelets will not aggregate in response to any concentration of adrenaline (Rand *et al.*, 2003).

Platelet Activator	Receptor	Effects on platelet	References
Primary agonist			
Collagen	GPIb GPIIb-IIIa GPIa/IIa GPIV	•Activation of GPIIb/IIIa •Release of ADP and thromboxane A2 •Platelet recruitment and aggregation •Induction of pro-coagulant activity via release of Ca^{2+}	(Jung and Moroi 2000)
von Willebrand factor		•Activation of GPIIb/IIIa •vWF-mediated platelet aggregation	(Yardumian, *et al* 1986)
Secondary agonist			
TXA_2	TPα	Platelet recruitment and aggregation to a primary platelet plug (TPα)	(Linder, *et al* 1979)
ADP	$P2Y_1$ $P2Y_{12}$	•Platelet shape change and transient aggregation ($P2Y_1$) •Sustained irreversible aggregation ($P2Y_{12}$) •Platelet recruitment to sites of injury, expression of P-selectin and release of TXA_2 ($P2Y_1$ and $P2Y_{12}$) •Induction of procoagulant activity and aggregation ($P2Y_{12}$)	(Mangin, *et al* 2004)
Thrombin	PAR-1 PAR-4	•Platelet aggregation (PAR-1) •Release of ADP, TXA_2 (PAR-4), serotonin (PAR-1) and adrenaline (PAR-1) •Activation/mobilisation of P-selectin and CD40 ligand (PAR-1) •Induction of platelet procoagulant activity (PAR-1)	(Ramström, *et al* 2008)
Adrenaline	α_2-adrenergic	•enhances activation initiated by other agonists	(Dunlop, *et al* 2000)

Table 2: *Primary and secondary platelet agonist, receptors and functions in platelet activation.*

1.2 PLATELET FUNCTION TESTING

Platelet function is measured clinically with a variety of techniques such as bleeding time, platelet aggregometry that measures platelet aggregation after stimulation with different agonists and obstruction of blood flow through a filter impregnated with different agonists, as in the case of the PFA-100 (Schwartz *et al.*, 2002). However, the most common method of assessing platelet function has been the measurement of platelet aggregation in citrated platelet rich-plasma (PRP) by turbidometry (Rand *et al.*, 2003). In a traditional aggregometry method, a minimum of 500µL of PRP in a cuvette in an aggregometer is warmed to 37^0C with rapid stirring followed by the detection of light transmission through PRP by photometer. Upon addition of an aggregating agent (e.g. collagen, ADP, arachidonic acid, adrenaline, the thromboxane analogue U46619, the PAR-1 activating peptide TRAP (e.g SFLLRN), the platelets change from their disc shape to a more rounded form with pseudopods, resulting in a transient, small decrease in light transmission that is followed by an increase as the platelets aggregate. Measurements include the rate and extent of the increase in light transmission. However, there is also limitation on platelet aggregometry because its sensitivity is limited to large aggregates formation whilst the formations of a few platelet aggregate are not detected (Cox, 1998).

Aggregometry is the gold standard for monitoring the effects of antiplatelet drugs such as aspirin, the thienopyridines and GPIIb/IIIa antagonists. By using platelets pre-labelled with radiolabeled serotonin, secretion of dense granule contents can be determined in

conjunction with aggregometry. Lumiaggregometry can also be used to measure the aggregation and secretion of the dense granule ATP simultaneously. Impedance aggregometry is used to measure platelet aggregation in anticoagulated whole blood. Other methods of measuring platelet function are rapid platelet function analyzer, platelet function analyzer (PFA-100).

1.3 THE ROLE OF PLATELETS IN ATHEROSCLEROSIS

Platelet plays a major role in the pathophysiology of atherosclerosis through their effects on inflammation. Despite their roles in modulating inflammation, platelets are directly involved in thrombosis and subsequent acute vascular events including acute coronary syndromes, ischaemic strokes and symptomatic peripheral arterial disease. Under pathological conditions, platelet aggregate formation, i.e. a thrombus, rapidly occurs within vasculature as a response to events such as plaque rupture (Willoughby *et al.*, 2002). The formation of thrombus may partially or totally occlude a vessel, resulting in the disruption of blood flow and tissue ischemia or necrosis. For this reason, development and clinical presentation of thrombi are often correlated with atherosclerotic disease. In addition, unstable angina as well as myocardial infarction (MI) may be the direct pathological result of thrombi in coronary arteries (Frishman *et al.*, 1995). Previous studies have shown a platelet hyperaggregable state in subjects who have various risk factors that are associated with atherosclerosis or coronary artery diseases, including diabetes mellitus, hypercholesterolemia, hypertension, and smoking (Willoughby *et al.*, 2002).

Endothelial dysfunction can be caused by high levels of circulating modified LDL-cholesterol, physical shear stress, free radicals, toxins from smoking, vasoactive amines, and infectious micro-organisms, results in a breakdown in the anti-inflammatory and anti-thrombotic properties of the endothelium that normally maintain cardiovascular homeostasis (Brydon *et al.*, 2006). At the site of vascular lesions, extracellular matrix proteins like von Willebrand factor (vWF) and collagen are exposed to the blood. Platelets adhere to vWF via the membrane adhesion receptor GPIb-V-IX and to collagen via GPVI Figure (1.2). This results in platelet activation and transformation of the integrin receptors αIIbβ3 (GPIIb-IIIa, fibrinogen receptor) and α2β1 (collagen receptor) (Nieswandt *et al.*, 2003), which firmly bind to the respective extracellular matrix components. Subsequently, platelets spread and form a surface for the recruitment of additional platelets via fibrinogen bridges between two αIIbβ3 receptors. This is followed by the release of adhesive and pro-inflammatory factors, which include pro-inflammatory cytokines, chemokines, vasoactive amines, and growth factors permits recruitment of leukocytes, lipids, smooth muscle cells, fibroblasts, and platelets to the arterial wall (Brydon *et al.*, 2006).

Atherosclerosis, the combined end-result of genetic modifiers, environmental factors, and spontaneous cellular-molecular events is a chronic disease that affects only medium to large sized arteries, primarily the coronary and cerebral arteries and aorta (Frishman *et al.*, 1995). Dysfunctional endothelial cells promote an increase of adhesiveness to platelets as well as leukocytes and secrete procoagulant compounds as a result of expression of

adhesion molecules, chemokines, growth factors, and inflammatory mediators. Although atherosclerosis usually is not fatal, it can develop into more complex lesions known as atherosclerotic plaques. If such plaques rupture the progression of a complex thrombus formation is facilitated by tissue factor that activates the coagulation cascade leading to thrombin generation, a potent platelet agonist as an end product and fibrin formation (McNicol *et al.*, 2003). These platelet-rich thrombi acutely block the blood supply to vital organs causing ischemic injury e.g. heart and brain (Ruggeri, 2000).

1.4 ANTIPLATELET THERAPY

1.4.1 Aspirin

Acetylsalicylic acid, or aspirin, is a synthetic compound with antipyretic, analgesic, anti-inflammatory, and antiplatelet properties. The pharmacological effects of aspirin are mediated primarily through its interference with prostaglandin biosynthesis. Aspirin's inhibitory effect on prostaglandin biosynthesis is due to its ability to acetylate cyclooxygenase-1 (COX-1), resulting in the inhibition of thromboxane A_2 release from platelets and prostaglandin (PG) I_2 from endothelial cells (Israels *et al.*, 2006). Although aspirin irreversibly inhibits both COX-1 and COX-2, its inhibitory effect on COX-1 is approximately 170-fold greater (Vane *et al.*, 1998). Platelets lack the synthetic machinery to generate significant amounts of new COX thus aspirin inhibitory effect persists for the

lifetime of the platelet. However, aspirin is still considered as a weak inhibitor of platelets because it blocks only thromboxane-dependent platelet activation and aggregation.

1.4.2 GPIIb-IIIa antagonists

As the final common pathway underlying platelet aggregation is binding of the adhesive proteins fibrinogen and von Willebrand factor (vWf), the fibrinogen receptor known as intergrin αIIbβ3 (GPIIb-IIIa) is a target for antithrombotic therapy. The blockade of fibrinogen binding to GPIIb-IIIa is the mechanism of action for this group of antiplatelet agent (Israels *et al.*, 2006). There are only three drugs of this class that are licensed by the FDA for clinical use. These three agents are a humanised antibody, abciximab; a non-peptide, tirofiban; and a peptide, eptifibatide, based on a snake venom sequence and a mimetic of the γ-chain peptide. The major potential adverse events resulting from GPIIa-IIIb antagonist therapy are bleeding, with the incidence of severe bleeding appearing to be more significant with abciximab than other GPIIa-IIIb antagonists. Other side effects are thrombocytopenia and pseudo-thrombocytopenia.

1.4.3 ADP receptor antagonist

There are four commercially available ADP receptor antagonists of thienopyridines, clopidogrel and ticlopidine (Israels *et al.*, 2006) and more recently prasugrel and ticagrelor (Cattaneo 2011). Clopidogrel, an irreversible $P2Y_{12}$ ADP receptor antagonist is well

established drug in clinical use for the treatment of peripheral artery disease and acute coronary syndrome as well as secondary prevention of ischemic stroke, vascular death and myocardial infarction while prasugrel, which acts similarly, and ticagrelor, which is a reversible inhibitor, have more recently become available (Pfefferkorn *et al.*, 2008).

1.4.4 Dipyridamole

Dipyridamole inhibits adenosine uptake in to erythrocytes and endothelial cells. This increases plasma adenosine levels, so permitting more binding of adenosine with platelet adenosine receptors (Chakrabarti *et al.*, 2008). This leads to increase in the platelet levels of cAMP and cGMP, and aggregation is inhibited.

Antiplatelet effects	Antiplatelet Drugs	References
Irreversibly acetylating COX and inhibits generation of TXA_2	Aspirin (75mg once daily)	(Israels and Michelson 2006)
ADP receptor antagonist	Ticlopidine (250mg twice daily) Clopidogrel (75mg onve daily)	(Schror, 1993), (Berglund *et al.*, 1998), (Collet *et al.*, 2011)
GPIIb/IIIa antagonists	Abciximab (bolus 0.25 mg/kg body weight, infusion 10 μg per min for 12 h) Tirofiban (bolus 10 μg/kg, infusion 0.15 μg/kg per min for 72 h) Eptifibatide (bolus 180 μg/kg, infusion 2 μg/kg per min for 72 h)	(Neumann *et al.*, 2001)
Adenosine reuptake inhibitor	Dipyridamole (200mg twice daily)	(Chakrabarti *et al.*, 2008)

Table 3: *Current antiplatelet drugs and mechanism of action*.

1.5 REACTIVE OXYGEN SPECIES AND ANTIOXIDANT DEFENSE SYSTEM IN PLATELETS

Reactive oxygen species (ROS) are generated in nearly all tissues in animals, plants and microbes and occur as byproducts of other biological reactions. Basically, ROS are oxygen-derived small molecules that include superoxide anion ($O_2^{\bullet -}$), hydroxyl ($^{\bullet}OH$), and also hydrogen peroxide (H_2O_2). Production of ROS as byproducts occurs with monoxygenases and dehydrogenases such as cytochrome P450 enzymes, xanthine oxidase/dehydrogenases, COX, LOX, and NADPH oxidase (Plumb *et al.*, 2005). ROS are crucially involves in many biological processes such as intracellular signalling in apoptosis and immunity, however, high levels of ROS or insufficiency in its removal will initate damages to tissues (Mates *et al.*, 1999). The enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), and glutathione (GSH) (Mates *et al.*, 1999).

Intracellular enzymatic antioxidant defenses eliminate superoxide and peroxides before they react with metal catalysis to form more reactive species. SOD is the antioxidant enzyme that catalyses the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2 that will be destroyed by CAT or GPX reactions (Fridovich, 1995). In humans, there are three forms of SOD: cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD (EC-SOD) (Sun *et al.*, 1995). Catalase protects cells from hydrogen peroxide that is produce in cells thus maintain the balance of oxidative stress in the adaptive response in cells.The most essential antioxidative defense mechanisms is

glutathione metabolism, by which GPX (80 kDa) catalyses the reduction of hydroperoxides using GSH to protect mammalian cells against oxidative damage (Mates *et al.*, 1999).

It has been shown that SOD activates human platelets exposed to subtreshold concentrations of AA and collagen (Iuliano *et al.*, 1991). Moreover, another study also found that in collagen-stimulated platelet, burst of hydrogen peroxide is important during aggregation process (Pignatelli *et al.*, 1998). ROS generation, Ca^{2+} mobilization and platelet aggregation were significantly greater in platelets from diabetic donors than in controls (Alexandru *et al.*, 2008). In contrast, platelet concentrations of GSH-Px, SOD and catalase activities are not changed in the whole group of diabetic patients in comparison to healthy subjects, suggesting that antioxidative enzymatic defence in blood platelets may have a minor role in affecting the modifications of haemostasis as well as the process of lipid peroxidation commonly observed in these patients (Seghieri *et al.*, 2001). Interestingly, an *in vivo* study found that acute exercise at 65% maximal oxygen uptake leads to platelet hyperreactivity which could be related to oxidative stress and/or TxA_2 pathway induced by exercise (Ficicilar *et al.*, 2003).

1.6 LIPOXYGENASE PATHWAY

Arachidonic acid (AA) released from platelet membrane phospholipid is metabolised through both cyclooxygenase (COX) and lipoxygenase (LOX) pathways with eicosanoids being produced as the end products. The influences of COX products, prostanoids, on platelet function have been extensively defined. On the other hand, the influences of hydroxyl derivatives synthesised from LOX pathway on platelet activation is not well understood, as different researchers report different findings. 12-LOX isoforms are named after the cells in which the enzyme isoforms were originally discovered; platelet, leukocyte and epidermis. In platelets, 12-lipoxygenases (12-LOX) catalyses dioxygenation of AA to the primary product, 12*S*-hydroperoxyeicosatetranoic acid (12S-HPETE) (Yoshimoto *et al.*, 2002). Then, 12(S)-HPETE is subsequently reduced to 12*S*-hydroxyeicosatetranoic acid, 12(S)-HETE, by glutathione-dependent peroxidase, and this is the major product of platelet 12-LOX.

There is relatively limited understanding of the function of 12-LOX despite its wide distribution in various tissues. For instance, myeloproliferative disorders are usually associated with thrombocythemia that often leads to bleeding complications. Reduced platelet 12-LOX activity is also found in these patients, associated with decreased platelet 12-LOX mRNA (Matsuda *et al.*, 1993). There has been a report of increased 12-LOX activity in the platelets of spontaneously hypertensive rats supporting previous evidence of an involvement of 12(S)-HETE in the pathogenesis of atherosclerosis (Chang *et al.*, 1985). AA

metabolism produces reactive oxygen species such as free radicals and oxidised lipid intermediates which are responsible for DNA damage and the generation of mutations. *In vitro* studies using cancer cell lines have demonstrated that LOX inhibitors are more potent than COX inhibitors at reducing cancer cell proliferation (Hong *et al.*, 1999). It has also been suggested that 12-LOX plays an essential role in regulating cell proliferation and apoptosis, with 12-LOX mRNA being highly expressed in various cancer tissues such as prostate and breast cancer (Yoshimoto *et al.*, 2002). The role of 12-LOX in cell growth and degeneration has been supported by another study that found a decrease in glutathione led to the neuronal activation of 12-LOX (Li *et al.*, 1997). Thus, neuronal degeneration will occur as a result of peroxide generation and increased calcium influx following 12-LOX activation.

1.7 NADPH OXIDASE

NADPH oxidase is composed of seven members, Nox1, Nox2, Nox3 and Nox4 which are formed with the small membrane bound subunit p22phox whilst the other three are calcium-dependent, Nox5, Duox1 and Duox2 (Ambasta *et al.*, 2004). However, it appears that only Nox4 requires no activator, constitutively active and expressed ubiquitously in vascular cells (Brandes *et al.*, 2008). In contrast with Nox4, Nox1 and Nox2 requires activation by cytosolic subunits which involves translocation of p67phox and p47phox to form complex with Nox1 and Nox2 (Bedard *et al.*, 2007). The functions of each NADPH oxidase are dependent on their localisation as all vascular oxidases generate ROS (Table

4). In vasculature, Nox1 is activated mainly by growth factors in an agonist-dependent manner, such as angiotensin II, platelet-derived growth factors, basic fibroblast growth factor as well as cytokines and mechanical stress (Brandes *et al.*, 2008). In addition, Nox2 is stimulated by vascular endothelial growth factor (VEGF) (Tojo *et al.*, 2005). Although Nox4 is constitutively expressed in vascular cells, overexpression of Nox4 can occur in vascular injury and can be down regulated by cytokines, angiotensin II or PDGF (Lassegue *et al.*, 2001).

The important role of NOX in vasculature was noted to be ROS-dependent increase in blood pressure as demonstrated in vivo in Nox1 knockout mice which develop less angiotensin II-induced hypertension (Matsuno *et al.*, 2005). In addition, ROS formations by vasculature NOX are thought to interact with NO, therefore limit NO bioavailability and elicits endothelial dysfunction (Jung *et al.*, 2004). The NOX-derived ROS also has been implicated in various vascular diseases such as vascular diabetes complications. Nox2 has been associated with overproduction of ROS in diabetes, and deletion of Nox2 gene showed an improvent of postischemic neovascularisation in mouse with Type I Diabetes Mellitus (Ebrahimian *et al.*, 2006). In addition, Nox2-derived ROS also has been linked to atherosclerosis although more evidence is needed to support this (Bedard *et al.*, 2007).

NOX Isoform	Location	Function
NOX 1	-plasma membrane of vascular smooth muscle cells	-proliferation and migration of smooth muscle cells -contributes to vascular disease
NOX 2	-plasma membrane of endothelium	-endothelial cell tube formation and angiogenesis -overexpression of Nox2 associates with atherosclerosis
NOX 4	-intracellular of vascular cells and endothelium	-differentiation of myofibroblast and smooth muscle cells -regulation of basal oxidative stress in cells
NOX 5	-endothelium	-unknown

Table 4: *Composition of NADPH oxidase in vasculature*. At least 3 different Nox isoforms are expressed in vascular cells.

1.8 RESEARCH OBJECTIVES

The main aims of my research is to optimise, modifies and validate of traditional platelet aggregometry in a 96-well plate format to determine mouse platelet activity, settings of combination agonists and also *in vitro* and *in vivo* investigation of the effects of COX-1 and COX-2 pathways on platelet reactivity. In platelets, superoxide anion and H_2O_2 are constitutively released with enhanced production following platelet stimulation by thrombin or collagen, or by immunological stimuli (Del Principe *et al.*, 2009). Previous findings showed that phagocyte NADPH oxidases apparently produce superoxide anions as primary products, rather than as byproducts (Brandes *et al.*, 2008). Therefore, I investigated the possible role of non-cyclooxygenase pathways, which are 12-lipoxygenase and NADPH oxidase on platelet function using a combined platelet aggregation and adhesion assay in a 96-well plate format. By using this technique, dietary influences of dark chocolate consumption on platelet function in midline hypertensive patients will also be determined.

CHAPTER TWO:

OPTIMISATION IN MEASUREMENT OF HUMAN AND MOUSE PLATELET USING 96-WELL PLATE FORMAT

2.1 Introduction

Platelet function tests traditionally are utilised mainly for the diagnosis and management of patients presenting with bleeding problems rather than thrombosis. However, new and improved existing platelet function tests are now increasingly used to monitor anti-platelet therapy in patients as well as to identify patients at risk of arterial disease. Light transmission aggregometry is still regarded as the gold standard of platelet function testing. In this study, traditional light transmission aggregometry (LTA) was modified to a 96-well plate format to evaluate platelet aggregation as well as adhesion using platelet-rich plasma. This method allows examination of response to many agonists at a range of concentrations on platelets at the same time point, thus more information can be collated about many different aspects of platelet function. For example, different agonists activate platelets through different pathways. Thus, changes in platelet aggregation induced by particular agonists may indicate the involvement of different pathways in changes in platelet function or the effects of different antiplatelet agents. Modification of the same method by reducing the samples volume to use half-area 96-well plates allows the measurement of platelet aggregation in samples from small laboratory animal, such as mice. It is important to note that in physiological conditions, platelets are activated not only by a single agonist but by multiple platelet activators. Therefore, to further explore this method some experiments employed combinations of agonists in the 96-well plate format.

2.2 Methodology

2.2.1 Blood Collection

2.2.1.1 Human Blood

Human platelets were obtained from whole fresh blood drawn from donors who had not consumed any medication for 10 days prior to the test. Healthy volunteers can be male or female aged 18-40 years old who are fit and healthy, not receiving current healthcare or have any known allergic to medicines. Blood was collected by venepuncture as 9 parts blood and 1 part anticoagulant. The anticoagulant used in this study was sodium citrate at 3.2% w/v or 0.105M.

2.2.1.2 Mouse Blood

Male C57B/6 mouse aged 8-10 weeks were acclimatised for a week before any procedure was performed. For each experiment 4-5 mice were used to obtain whole blood by terminal cardiocentesis. Mouse were anaethesised using inhaled halothane and further narcosis with a slowly rising concentration of CO_2, 1mL of C57B/6 mouse blood was withdrawn by surgical cardiac puncture with a 1 ml syringe and 23G needle into 0.1 ml of 100U/mL heparin.

2.2.2 Preparation of Platelet Rich and Platelet Poor Plasma

Human platelet-rich plasma (PRP) was obtained from blood by centrifugation at 1100 rpm for 15 minutes at room temperature. The cloudy yellow supernatant containing the platelets was removed carefully with a disposable plastic pipette and placed into a clean polypropylene tube and capped. Care was taken not to disturb the WBC and RBC cell layers when removing the PRP. The prepared PRP was kept at 37^0C in a waterbath prior to use. Platelet-poor plasma (PPP) was prepared by further centrifugation of PRP at 15000 for 2 min using a microcentrifuge. PRP and PPP were used soon after the preparation or if not within 2 hours of preparation to avoid any spontaneous platelet aggregation and adhesion. Platelet counts were checked to ensure they were within the normal range which is 150-400 x 10^9/L.

To obtain mouse PRP, blood was diluted, 6:1, in by the addition of 200µl of 10U/mL heparin in each tube before being centrifuged at 900 rpm using a bench top microcentrifuge. Heparin was prepared to the desired concentration by further diluting the stock with Tyrodes Buffer containing 134mM NaCl, 2.9mM KCl, 1mM $MgCl_2$, 0.34mM Na_2HPO_4, 12mM $NaHPO_3$, 20mM HEPES and 5mM glucose, at pH 7.3. Then the plasma was taken out from the microcentrifuge including the 1/3 of blood remaining and transferred into a fresh microcentrifuge tube followed by centrifugation at 700 rpm for 5 minutes. The PRP was then removed into a new tube and diluted for platelet counting. Platelet counting was done by adding 10µl of PRP into 5mL of water followed by transferance of 10µl into a

haemacytometer to be counted under a microscope. PPP was obtained by further centrifugation at 15000 rpm for 2 minutes.

2.2.3 Platelet adhesion assay in 96-well plate using modified Bellavite method

Platelet adhesion assay in 96-well plate format using modified Bellavite method (Bellavite *et al.*, 1994) was carried out using pre-coated plates to determine the effects of coating upon platelet adhesion. To coat the plates, 2mg/ml of fibrinogen or human albumin were prepared in PBS before adding 100µl into each well. Plates then were left overnight at 4^0C and washed with 100µl of 0.9% saline prior to use. Initially prepared PRP was further diluted to ¼ into either dilution buffer or PPP respectively. Dilution buffer contained 145mM NaCl, 5mM KCl, 10mM HEPES, 0.5Mm $NAHPO_4$, 6mM glucose and 0.2% human serum albumin. 25µl of agonist, example collagen at concentration ranging from 0.1-30µM is added into each agonist well followed by 50µl diluted PRP. Plate was then incubated for 1 hour at room temperature on the bench. This was followed by washing the plate twice with 100µl 0.9% NaCl each well. During final washing, the plate was tapped a few times on a paper towel to remove any remaining saline. Once the plate was washed, 100% adhesion controls were prepared by centrifugation of 500µl PRP at 6000rpm for 2 minutes. The plasma was discarded and the pellet resuspended in 700µl assay buffer. The Buffer B was prepared by combining 16.2ml of distilled water, 5.2ml of 0.1M citric acid, 16.5ml of 0.1M sodium citrate and 40µl of Triton-X-100 with a final pH of 5.4. This was followed by preparation of assay buffer containing *p*-nitrophenol phosphate with addition

of 2 ml of 10x assay buffer (1 tablet of *p*-nitrophenol phosphate dissolved in 10ml of Buffer B) into 36ml of buffer B. Then, 100µl of 100% control adhesion was pipetted into control wells. For remaining wells, 100µl of assay buffer containing *p*-nitrophenol phosphate was pipetted into each well before 15 minutes incubation on rotary shaker. To stop the reaction, 100µl of 2M NaOH was added into each well, which turned the solution yellow if *p*-nitrophenol was present. Then, the plate was read at 405nm using a microplate reader. Background absorbance was subtracted from these values. The percentage of adhesion was calculated using formula; {(Absorbance of sample-Blank) / (Absorbance of control-Blank)} x 100.

2.2.4 Combined Platelet Aggregation and Adhesion Assay using 96-well plate format

Platelet aggregation was adapted into 96-well plate format starting by combining 100µl PRP in each well with 10µl of agonist or vehicle, phosphate buffer saline (PBS). All agonists used in these experiments were diluted in PBS. The final concentrations of agonists used were; ADP, U46619, thrombin and TRAP-6 amide 0.1, 0.3, 1, 3, 10 and 30µM; collagen 0.1, 0.3, 1, 3 .10 and 30µg/mL; adrenaline 0.001, 0.01, 0.1, 1, 10 and 100µM; ristocetin 0.2, 0.3, 0.6, 1, 2 and 3mg/ml; arachidonic acid, 10µM to 1.6mM (Figure 2.1) (Table 5). All agonists were prepared freshly prior to use and kept on ice. Plate setup was designed so that the first row was dedicated for PPP, PRP and H_2O/buffer as a control. Then, the plate was quickly placed on a microplate reader with absorbance determined at 595nm and

read for 64 cycles, each cycle consisting of vigorous shaking for 7 seconds interval and reading for 8 seconds with temperature setting at 37^0C; this resulted in a total assay time of approximately 16 minutes for each 96-well plate. To test inhibition of platelet aggregation and adhesion, PRP was pre-incubated for 30min before being added into a plate in the presence of agonists. For half-area plates used in human platelet and mouse platelet experiments the volumes used for the platelet aggregation procedures were reduced by half.

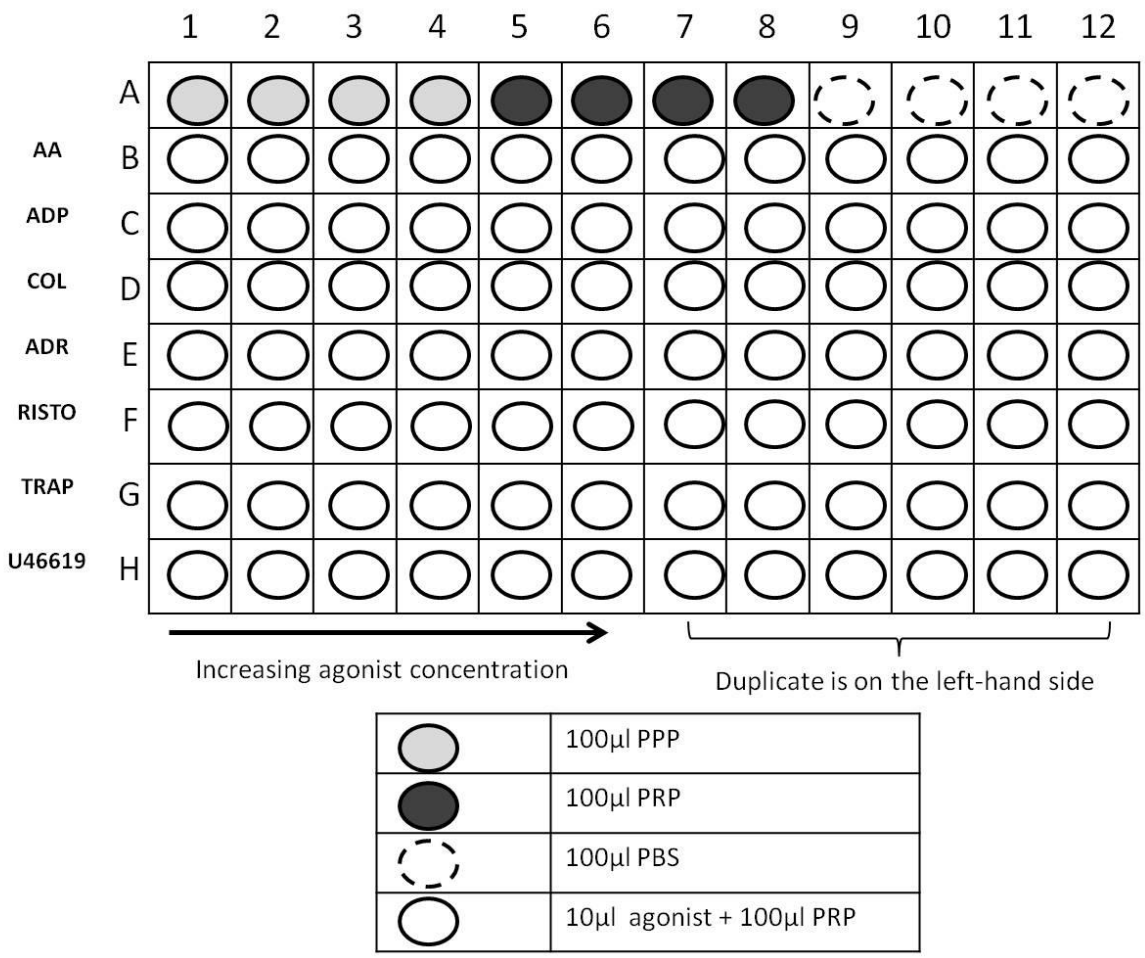


Figure 2.1: *Plate design for combined platelet aggregation and platelet adhesion in 96-well plate.*

Agonist	Concentration
Arachidonic acid (mM)	0.03, 0.1, 0.3, 1, 3, 10
ADP (µM)	0.1, 0.3, 1, 3, 10, 30
Adrenaline (µM)	0.001, 0.01, 0.1, 1, 10, 100
Collagen (µg/ml)	0.1, 0.3, 1, 3, 10, 30
Ristocetin (mg/ml)	0.2, 0.3, 0.6,1, 2, 3
TRAP-6 (µM)	0.1, 0.3, 1, 3, 10, 30
U46619 (µM)	0.1, 0.3, 1, 3, 10, 30

Table 5: *List of agonist and concentration used in combined platelet aggregation and adhesion in 96-well plate.*

For platelet adhesion determination at the end of the platelet aggregation tests, plates were emptied of PRP by inversion. Then, the wells were washed twice with 100µl of 0.9% saline. During final washing, the plate was tapped a few times on a paper towel to remove any remaining saline. Starts from this stage, platelet adhesion method was carried out as previously described in section 3.2.3. For studies of mouse platelet adhesion, all the volumes were reduced by half, as half-area plates were used. It is noteworthy to acknowledge that all the experiments beginning from human and mouse platelet aggregation and adhesion in half-area 96-well plates onward were to follow combined platelet aggregation and adhesion assay using 96-well plate format described in this section.

2.2.5 Combination of Agonists

For experiments examining the effects of combinations of agonists on platelet, the lowest three concentrations of collagen, TRAP-6 and adrenaline were used. The combinations for these experiments are summarised in the Table 6 below.

Combination	**Collagen**	**Adrenaline**	**TRAP-6**
		Single agonist	
COL	●		
ADR		●	
TRAP-6			●
		Dual agonists	
COL+ADR	●	●	
COL+TRAP-6	●		●
ADR+TRAP-6		●	●
		Triple agonists	
COL+ADR+TRAP-6	●	●	●

Table 6: *Summary of combinations of agonists for 96-well plate assays.* Each agonist was tested in three different concentrations both individually and in combination. Notes: Collagen, COL; adrenaline, ADR; and TRAP-6.

2.2.6 Investigation of platelet inhibition

To investigate the effects of prostacyclin (PGI_2), sodium nitroprusside (SNP), rosiglitazone and aspirin on platelet function, platelets were incubated with drug for 30 min at 37^0C prior to exposure to platelet agonists. Concentrations of drugs used were as follows: prostacyclin (100nM, 1µM and 10µM); SNP (0.001 and 0.1µM); rosiglitazone (10 and 100µM); and aspirin (0.1-300µM).

2.2.7 Thromboxane B_2 Determination by Radioimmunoassay

Plasma samples for measurement of TxB_2 were obtained by adding 10µl of 10mM diclofenac into each well at the end of platelet aggregation to stop TxA_2 production, followed by transfer into a fresh plate. Then, the plate was centrifuged at 3000 rpm for 15 minutes at 4^0C, the plasma removed and stored at -20^0C. The samples were thawed prior to use. Plasma samples were diluted at 1:5 with mix-Tris buffer. Then, either samples or standard concentrations ranging from 0.017 to 20ng/ml of TxB_2 were mixed with TxB_2 tracer and TxB_2 antibody before being incubated overnight at 4ºC. Separation of bound antibody from free antibody was achieved by adding 50µl of charcoal-Dextran buffer and centrifugation at 40°C for 10min at 3000rpm. Then, 100µl from each well was transferred into fresh white transparent bottom 96-well plates and sealed. Radioactivity was counted in a liquid scintillation counter.

2.2.8 Statistical Analysis

Pharmacological parameters were analysed by GraphPAD Prism 5.0 (San Diego, USA) software. ANOVA was performed when appropriate. Data are expressed as mean ± standard error of mean (S.E.M.). All experiments were performed in duplicates of at least n=4 unless stated.

2.3 Result

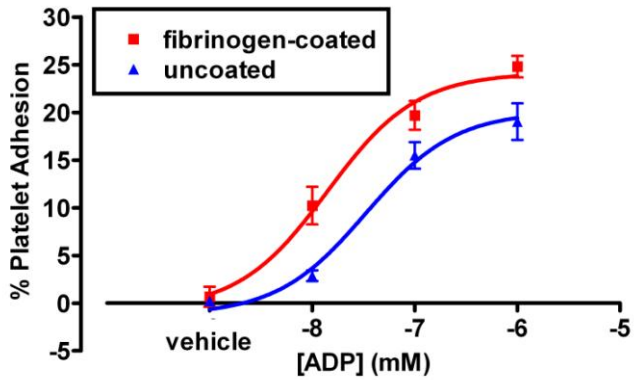
2.3.1 Optimisation of Platelet Adhesion in 96-Well Plates

PRP for use in these experiments was diluted 4-fold with dilution buffer prepared as described above. To study the adhesion of platelets in buffer-diluted PRP, 3 different types of plates were used; fibrinogen-coated, human albumin-coated and uncoated plates. As shown in Figure 2.2, platelet adhesion on fibrinogen-coated plates was higher as compared to non-coated, whilst coating with albumin reduced platelet adhesion as compared to non-coated plates.

ADP stimulated platelet adhesion in a concentration-dependent manner in both pre-coated and uncoated plates. However, platelet adhesion to fibrinogen-coated plates was higher than that to uncoated plates, with maximal adhesion being 24±1% compared to 20±2%, respectively. In contrast, platelets adhered significantly less to albumin-coated than uncoated plates. Since the fibrinogen coating increased platelet adhesion, samples of PRP diluted in PPP were further tested in fibrinogen-coated and non-coated plates in the presence of various agonists, including ADP, calcium ionophore A23187, collagen, arachidonic acid and adrenaline. All agonists tested in this experiment caused concentration-dependent increases in platelet adhesion that was greater in pre-coated than uncoated plates (Figure 2.3 and Figure 2.4).

i. Platelet adhesion of PRP diluted 1:3 in Dilution Buffer

a)



b)

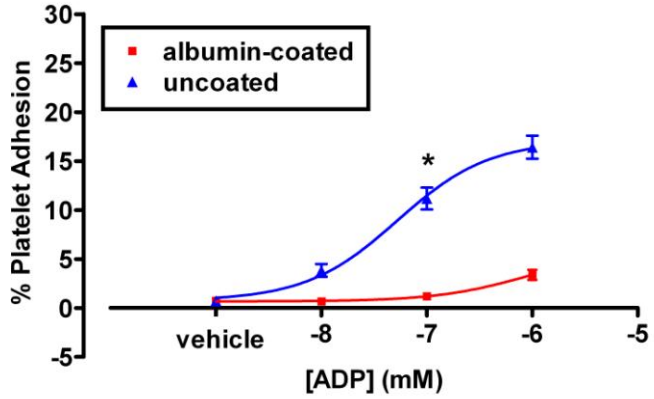


Figure 2.2: *Platelet adhesion of PRP diluted in dilution buffer in response to ADP on differently coated plates*. Platelet adhesion; a) on fibrinogen-coated and uncoated plates and b) on albumin-coated and uncoated plates. Data shown as mean ± S.E.M. mean from duplicate responses from four different individuals. * indicates $p<0.0001$ by two-way ANOVA.

ii. Platelet adhesion of PRP diluted 1:3 in PPP

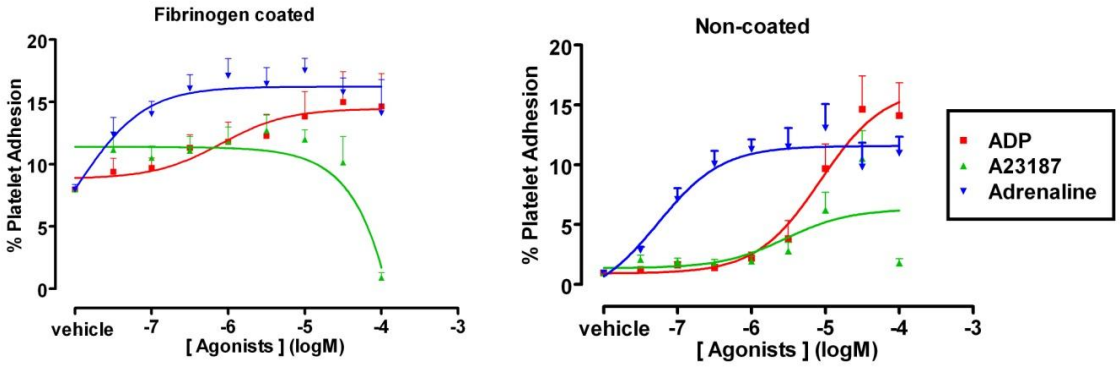


Figure 2.3: *Platelet adhesion of PRP diluted in PPP in response to ADP, calcium ionophore A23187, or adrenaline on differently coated plates*. Platelet adhesion a) on fibrinogen-coated plates and b) on uncoated plates. Data shown as mean ± S.E.M. mean from duplicate responses from four different individuals.

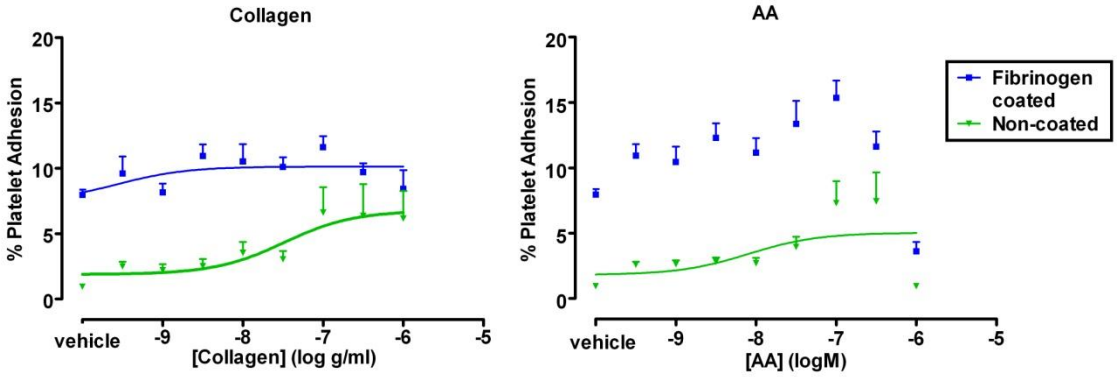


Figure 2.4: *Platelet adhesion of PRP diluted in PPP in response to collagen or arachidonic acid on differently coated plates*. Data shown as mean ± S.E.M. mean from duplicate responses from four different individuals.

2.3.2 Inhibition of Prostacyclin on Platelet Adhesion in 96-Well Plate

Since previous experiments demonstrated that this method is reliable to determine platelet adhesion in 96-well plate format following stimulation with platelet agonists, the effects of prostacyclin, a platelet inhibitor, on adhesion in response to ADP was determined for both albumin and fibrinogen-coated 96-well plates. As shown in the Figure 2.5, albumin-coated plates demonstrated lower platelet adhesion as compared to uncoated plates whilst the adhesion was greatest on fibrinogen-coated plates. No inhibition of platelet adhesion by prostacyclin was observed in albumin coated plates in respect of unstimulated platelets, however, a slight inhibition was shown in ADP-stimulated platelets at the highest three concentrations of prostacyclin. Addition of prostacyclin slightly reduced ADP-stimulated platelet adhesion to fibrinogen-coated plates, but not that of unstimulated platelets. For instance, after incubation with 100nM, 1μM and 10μM prostacyclin, platelet adhesion was decreased to 23±3%, 24±3% and 22±3% as compared to control vehicle, 26±3%. In unstimulated platelets the highest two concentration of prostacyclin slightly decreased the adhesion of platelets to fibrinogen-coated plates. For uncoated plates, a pattern of inhibition of platelet adhesion by prostacyclin was found in both albumin and fibrinogen-coated plate experiments although unstimulated platelets displayed higher levels of inhibition by prostacyclin. For example, in fibrinogen-coated plate experiments, prostacyclin at 100nM, 1μM and 10μM inhibited unstimulated platelet adhesion to 19±7%, 18±8% and 16±7% as compared to control vehicle, 21±5%.

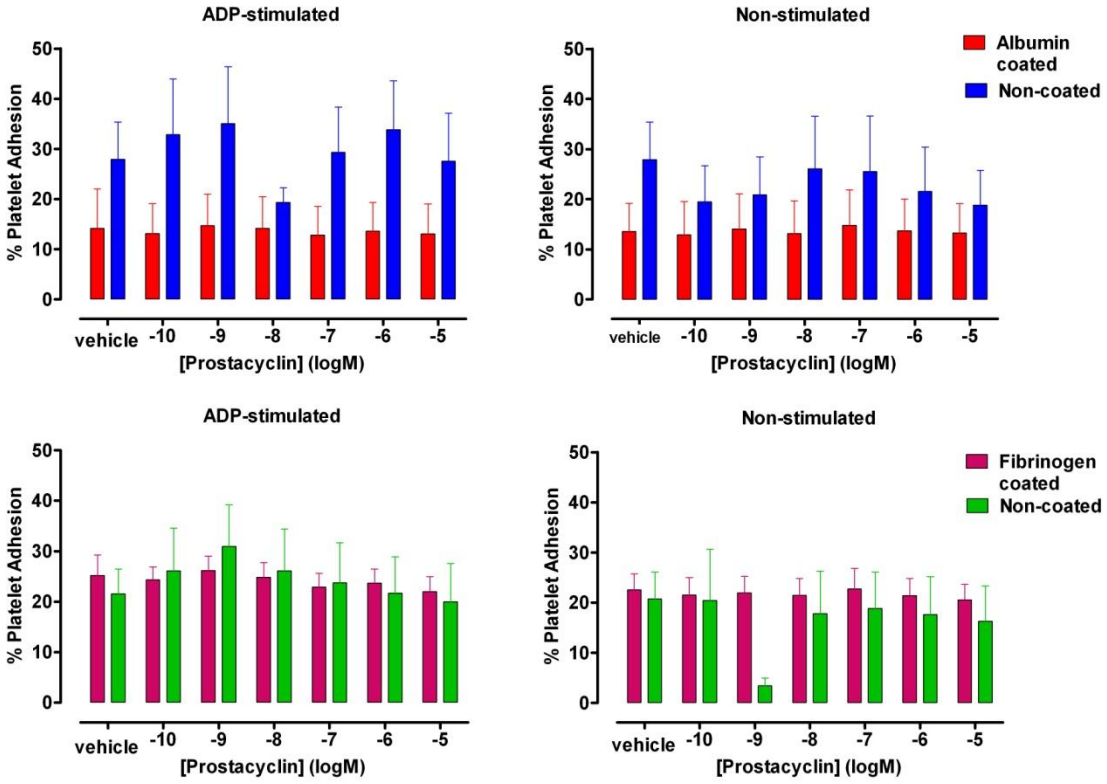


Figure 2.5: *Inhibition by prostacyclin of platelet adhesion.* PRP diluted in PPP was incubated with prostacyclin before addition to fibrinogen or albumin coated plate and stimulation with 10µM ADP or vehicle. Each value represent means ± S.E.M. (n=3-4).* indicates $p<0.05$ by one-way ANOVA as compared with control vehicle.

2.3.3 Inhibition by Sodium Nitroprusside on Platelet Adhesion in 96-Well Plate

Figure shows that an increased of platelet adhesion is observed following stimulation by several agonist (Figure 2.6). As compared to control (2±0%), platelet adhesion was increased to 8±2%, 5±3% and 12±3% ($p<0.01$) when stimulated with ADP, A23187 or adrenaline, respectively. As shown in Figure 2.7, incubation with SNP reduced the stimulated platelet adhesion in a concentration-dependent manner. For example, 1µM and 10µM SNP significantly inhibited stimulate platelet adhesion to 39±7% and 6±4%, for ADP; 76±7% and 48±5%, for adrenaline-stimulated; and, 76±13% and 43±12%, for arachidonic acid (*p<0.05*). SNP also inhibited calcium ionophore (A23187)-stimulated platelet adhesion to 68±9% and 42±5%, and slightly inhibited adhesion following stimulation by collagen.

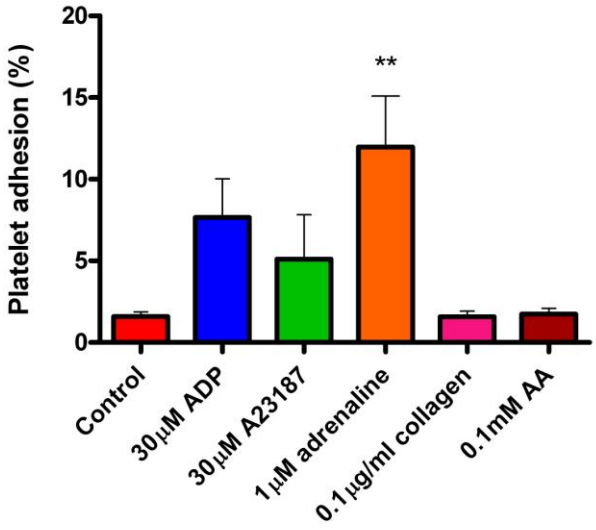


Figure 2.6: *Maximal response of platelet adhesion stimulated by various agonist as compared to control PBS.* Each value represent means ± S.E.M. (n=6). ** indicates $p<0.01$ by one-way ANOVA.

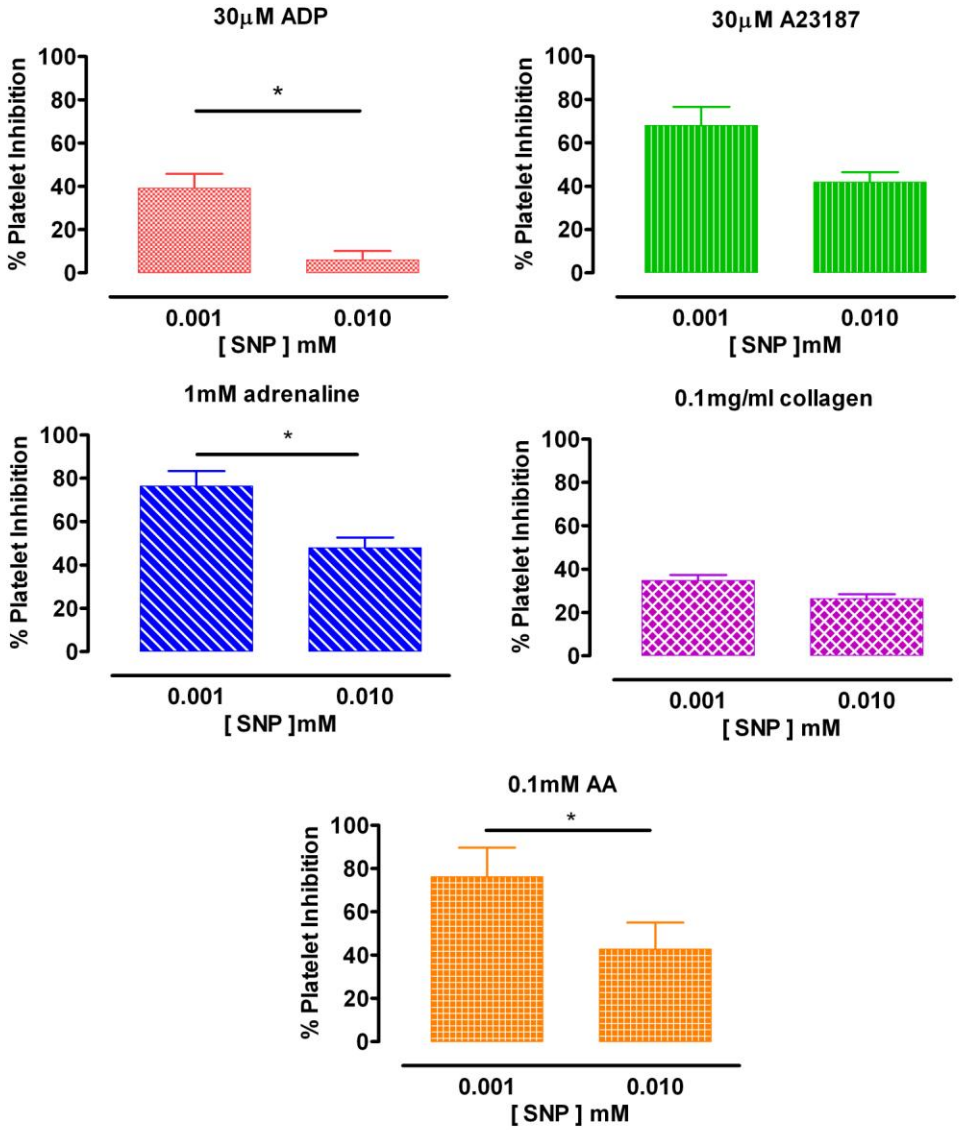


Figure 2.7: *Inhibition by sodium nitroprusside (SNP), 0.001μM and 0.01μM of platelet adhesion of PRP diluted in PPP.* Platelets were stimulated by various agonists including ADP, calcium ionophore A23187, adrenaline, arachidonic acid and collagen. Each value represent means ± S.E.M. (n=6). * indicates $p<0.05$ by two-way ANOVA.

2.3.4 Platelet Aggregation and Adhesion in Half-Area 96 Well Plate

The use of 96-well format to determine platelet function was further explored in a study of platelet aggregation and adhesion in half-area well plates in response to various platelet agonists. In these studies PRP was used without dilution into buffer or PPP. Platelet agonists used to induce platelet aggregation and adhesion in these experiments were arachidonic acid, ADP, adrenaline, TRAP-6, ristocetin, collagen and U6619. An example of absorbance read in stimulated PRP is shown in Figure 2.8. All platelet agonists induced platelet aggregation and adhesion of human platelets in concentration-dependent and time-dependent manners (Figure 2.9-2.10; Figure 2.14-2.15). The same method was used to determine aggregation and adhesion of mouse platelets (Figure 2.11-2.12; Figure 2.14-2.15). However, despite concentration-dependent platelet stimulation by most agonists mentioned above, mouse platelets did not show activation in response to thrombin or TRAP-6.

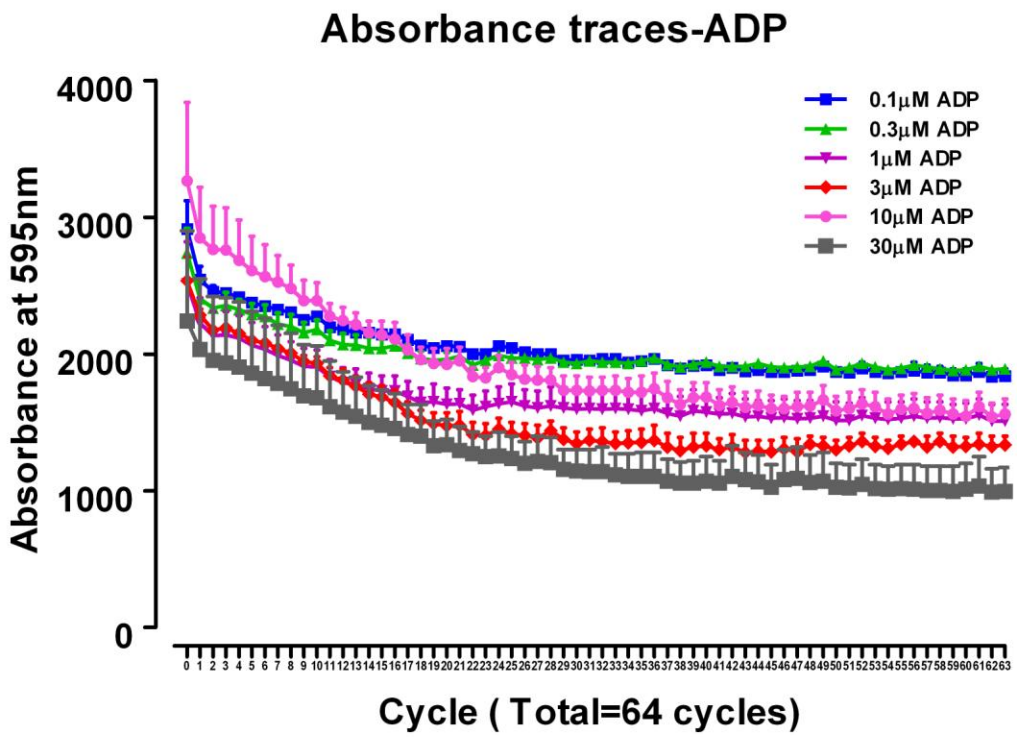


Figure 2.8: *Absorbance traces of ADP-stimulated human PRP in half-area 96-well plate*. For platelet aggregation in half-area 96-well plate, PRP is added into agonist plate, for example ADP; then read at 595nm for 64 cycles. Each cycle consisting of vigorous shaking for 7 seconds interval and reading for 8 seconds with temperature setting at 37^0C; this resulted in a total assay time of approximately 16 minutes for each 96-well plate. Data are mean ± S.E.M. from duplicate samples.

i. Human platelet aggregation and adhesion in half-area well plates

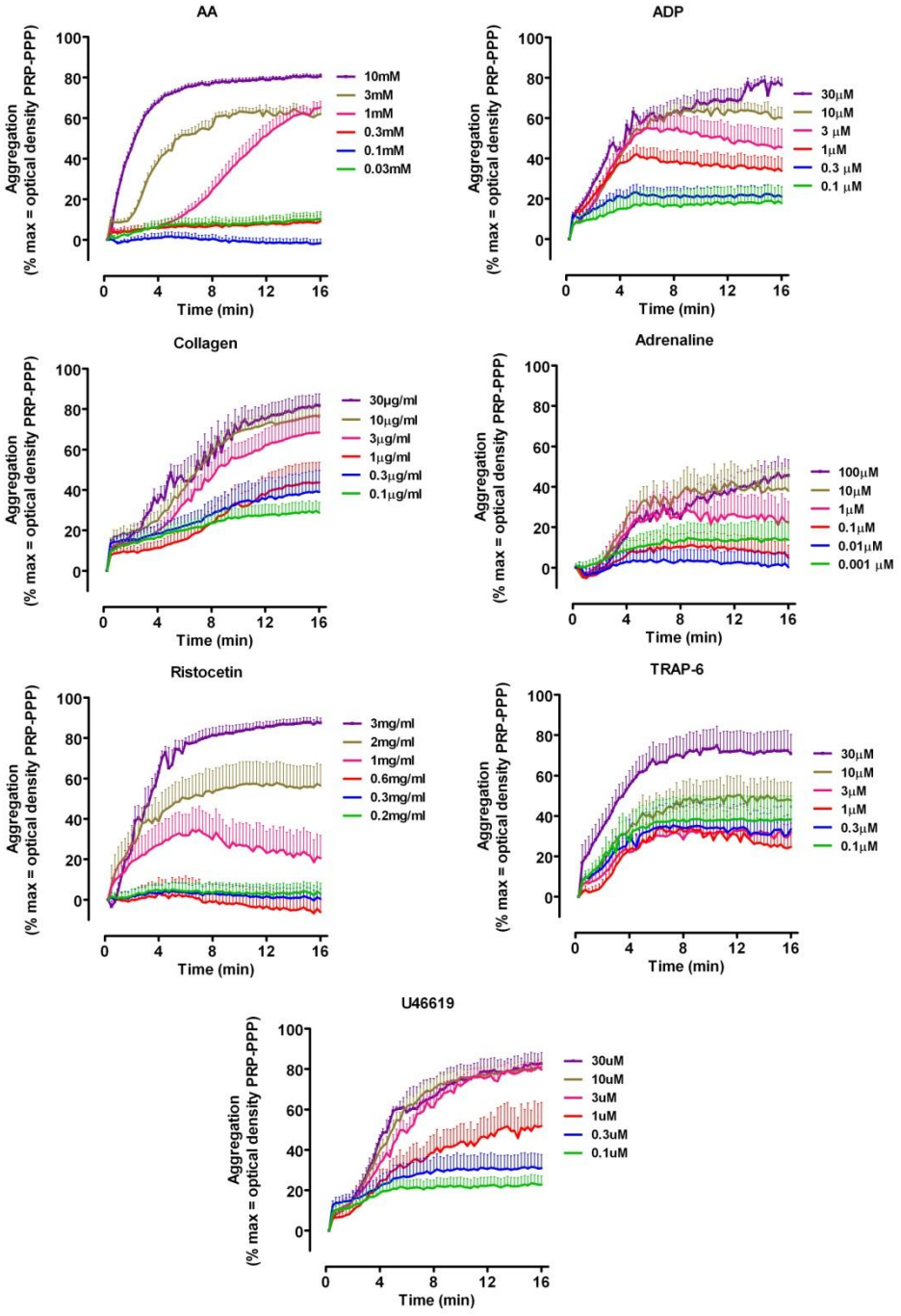


Figure 2.9: *Aggregation traces of human platelets in PRP stimulated by various agonists in half-area 96-well plates.* Platelets were stimulated with AA (0.03mM-10mM), ADP, TRAP-6, or thromboxane mimetic, U46619 (0.1-30μM), collagen (0.1-30μg/mL), adrenaline (0.001-100μM), and ristocetin (0.2-3mg/ml). Each value represents mean ± S.E.M. (n=4).

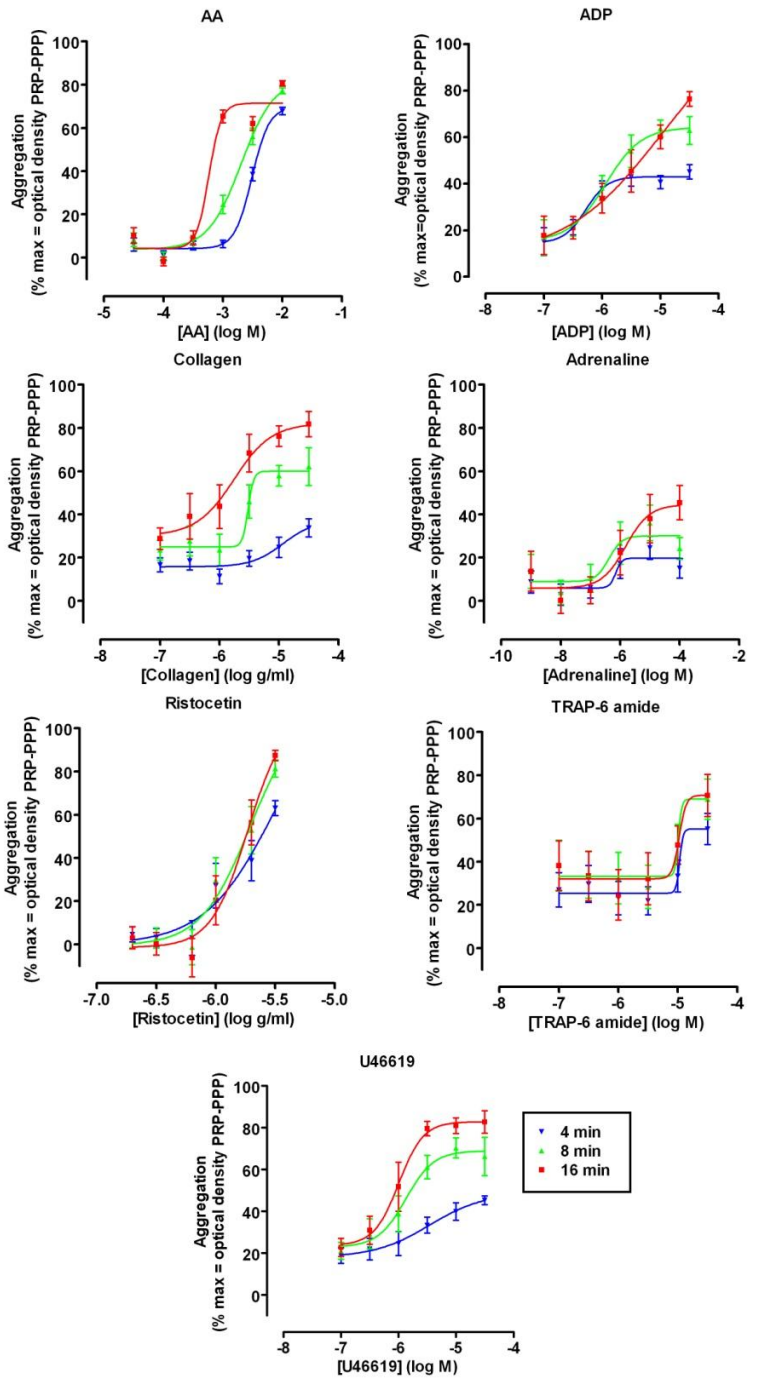


Figure 2.10: *Concentration response curves of human platelets stimulated by various agonists in half-area 96-well plates.* Platelets were stimulated with AA (0.03mM-10mM), ADP, TRAP-6, or thromboxane mimetic, U46619 (0.1-30µM), collagen (0.1-30µg/mL), adrenaline (0.001-100µM), and ristocetin (0.2-3mg/ml). Each value represent mean ± S.E.M. (n=4).

ii. Mouse platelet aggregation and adhesion in half-area small well plate

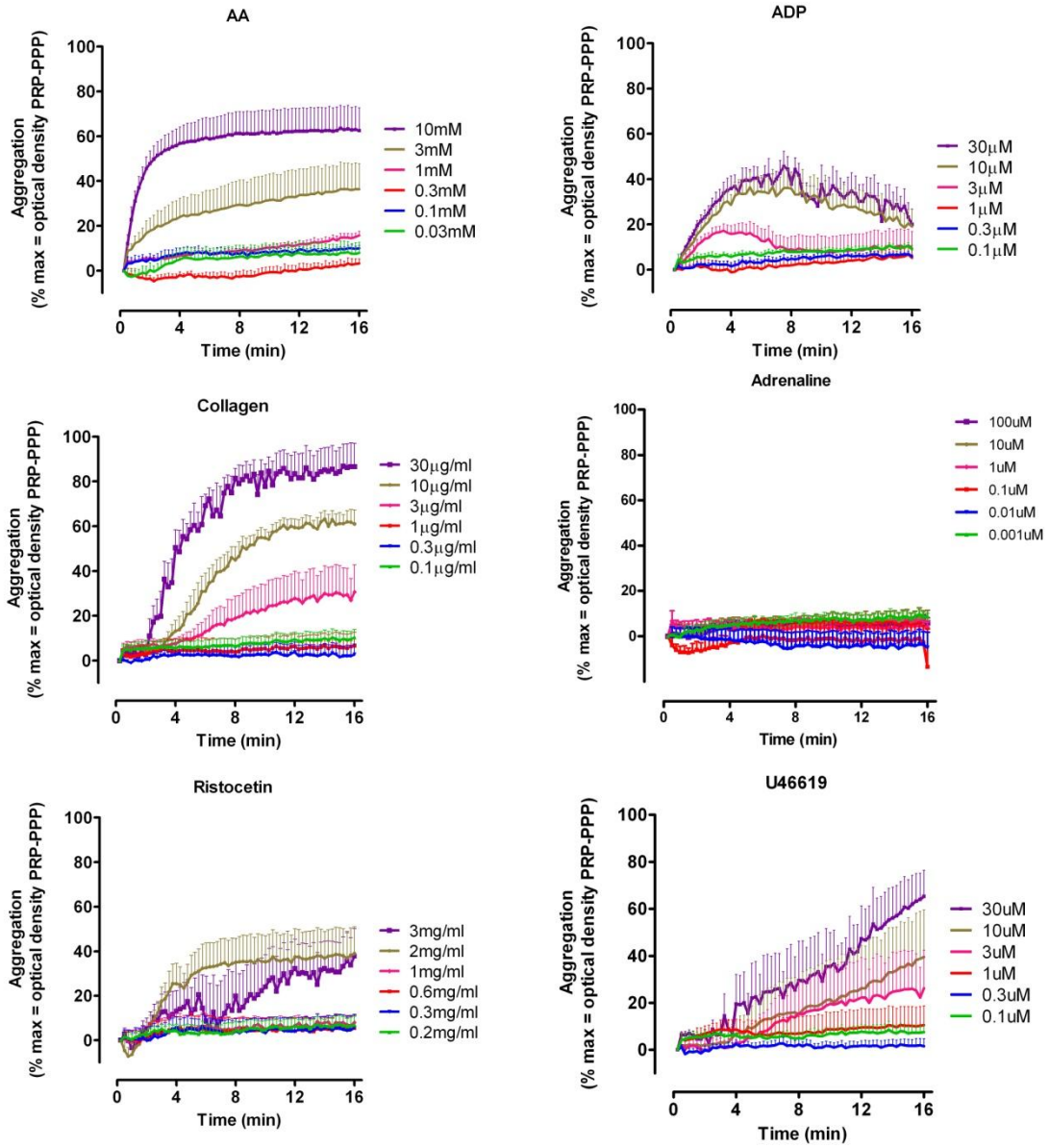


Figure 2.11: *Aggregation traces of mouse platelets stimulated by various agonists in half-area 96-well plates.* Platelets were stimulated with AA (0.03mM-10mM), ADP, or thromboxane mimetic, U46619 (0.1-30μM), collagen (0.1-30μg/mL), adrenaline (0.001-100μM), and ristocetin (0.2-3mg/ml). Each value represents mean ± S.E.M. (n=4).

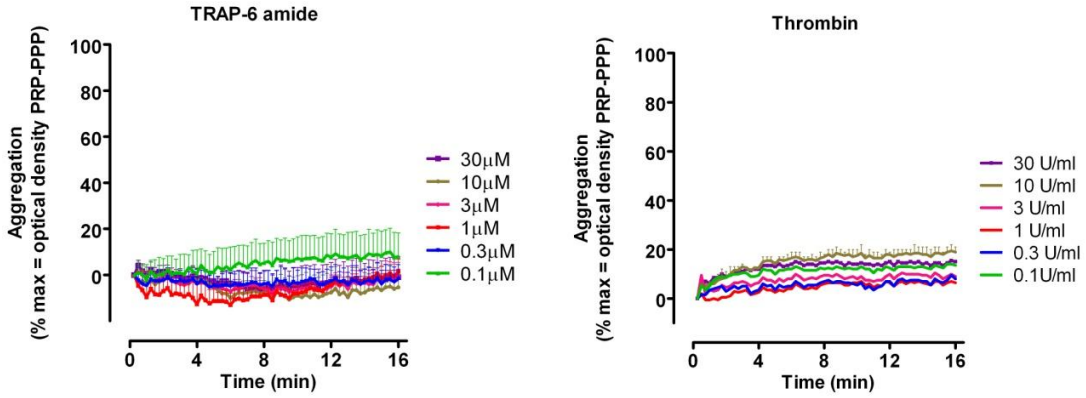


Figure 2.12: *Aggregation traces of mouse platelets stimulated by TRAP-6 and thrombin in half-area 96-well plates.* Each value represents mean ± S.E.M. (n=2).

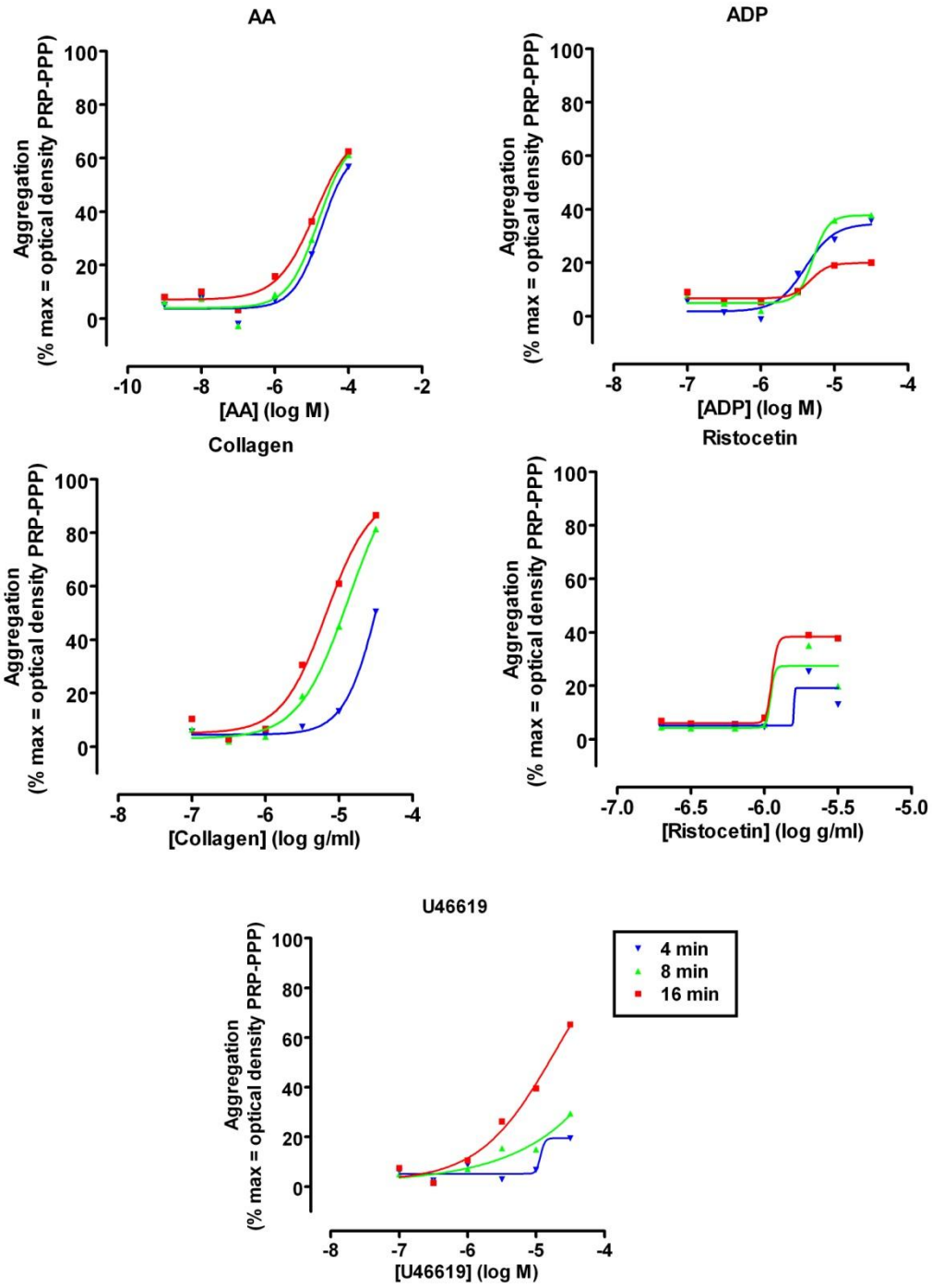


Figure 2.13: *Concentration response curves of mouse platelets stimulated by various agonists in half-area 96-well plates.* Platelets were stimulated with AA (0.03mM-10mM), ADP, or thromboxane mimetic, U46619 (0.1-30µM), collagen (0.1-30µg/mL), adrenaline (0.001-100µM), and ristocetin (0.2-3mg/ml). Each value represents mean ± S.E.M. (n=4).

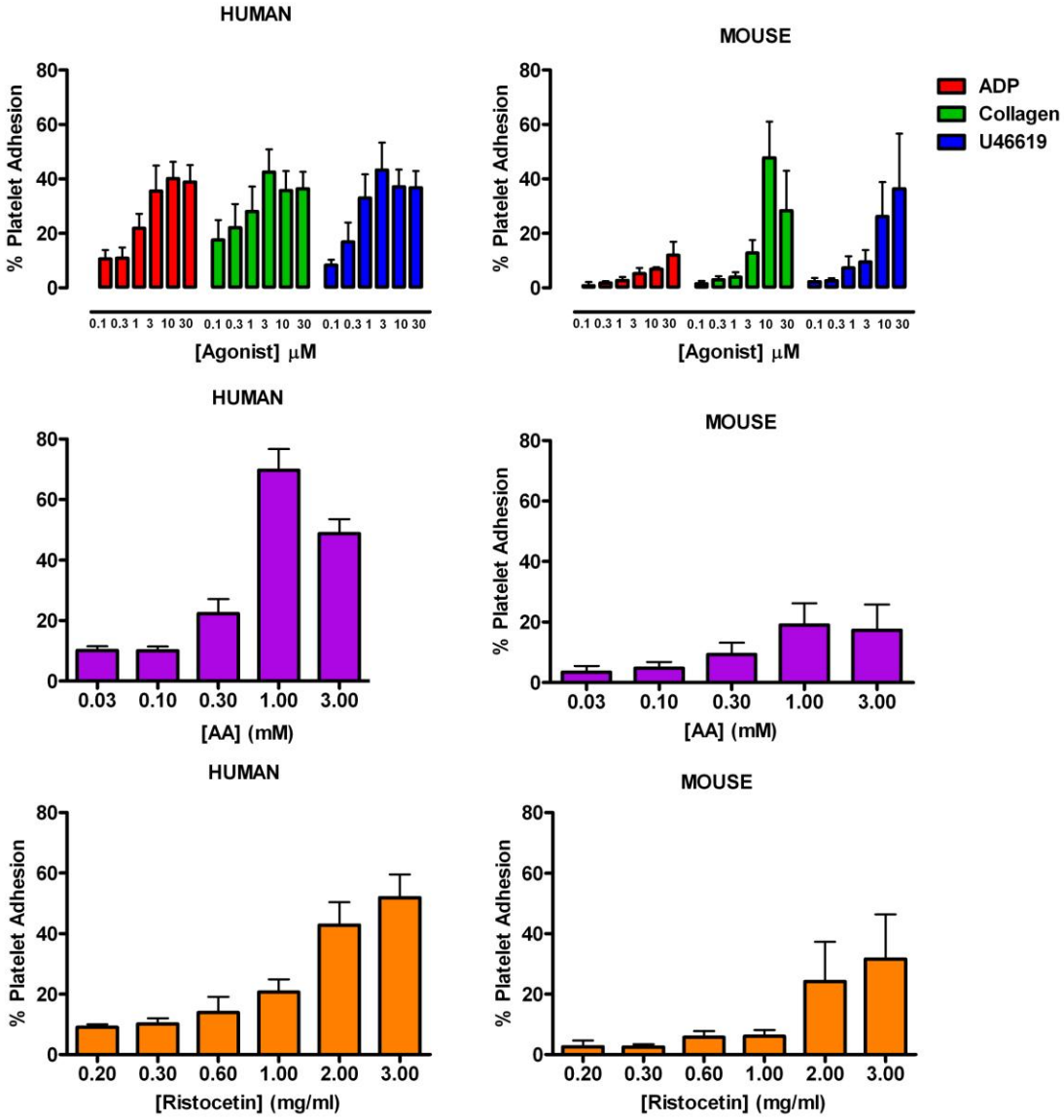


Figure 2.14: *Adhesion of human and mouse platelets stimulated by various agonists in half-area 96-well plates.* Platelets were stimulated with AA (0.03mM-3mM), ADP, or thromboxane mimetic, U46619 (0.1-30μM), collagen (0.1-30μg/mL), adrenaline (0.001-100μM), and ristocetin (0.2-3mg/ml). Each value represents mean ± S.E.M. (n=4).

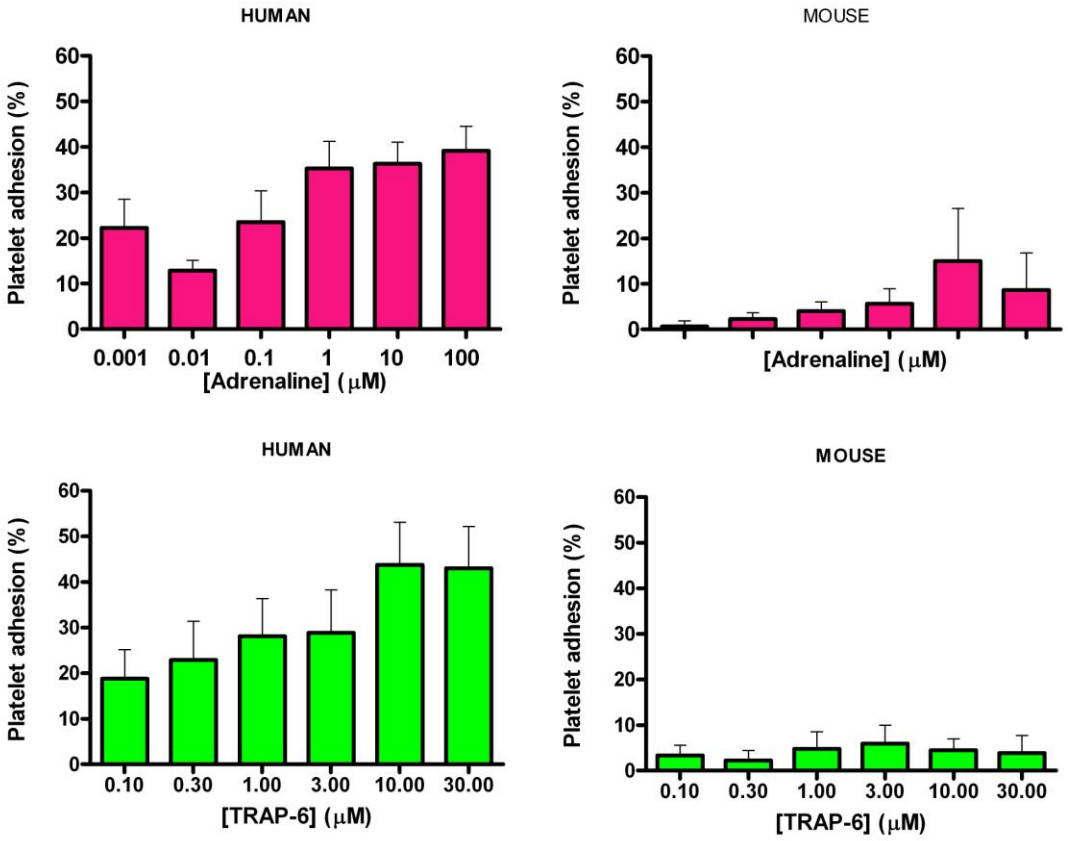
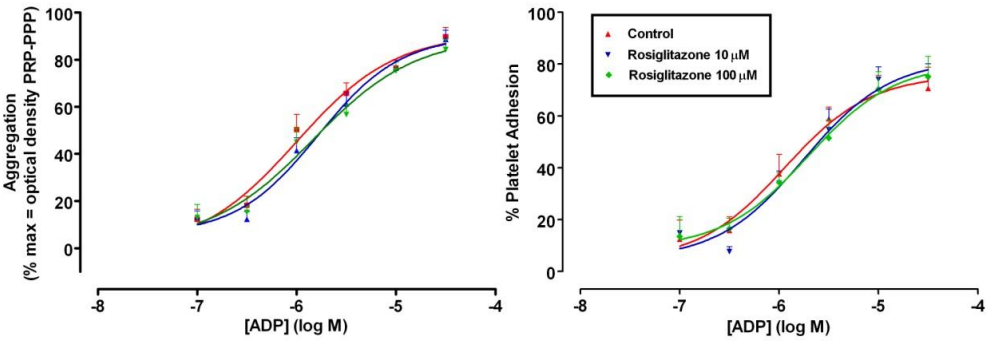


Figure 2.15: *Adhesion of human and mouse platelets stimulated by adrenaline and TRAP-6 in half-area 96-well plates.* Platelets were stimulated with TRAP-6 (0.1-30μM), and adrenaline (0.001-100μM). Each value represents mean ± S.E.M. (n=4).

2.3.5 Effects of Rosiglitazone on Human Platelet Aggregation and Adhesion Stimulated by ADP and Collagen

Rosiglitazone did not affect platelet aggregation or adhesion at either 10 or 100μM.

i) ADP



ii) **Collagen**

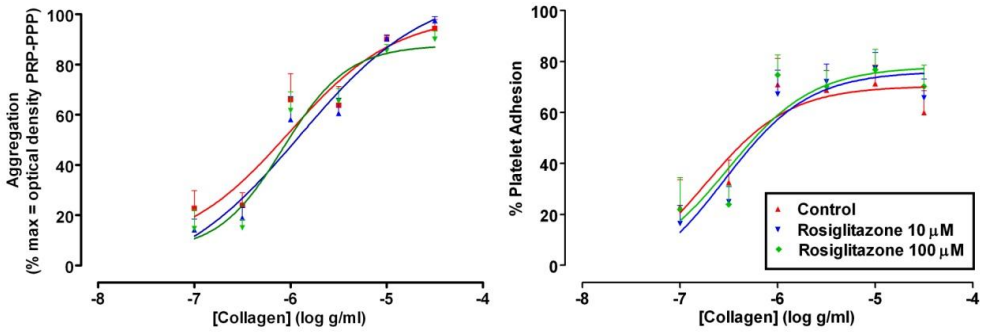


Figure 2.16: *Effects of rosiglitazone on aggregation stimulated by ADP or collagen.* Human PRP was incubated with 10μM and 100μM rosiglitazone before addition of (i) ADP and (ii) collagen. Each value represent means ± S.E.M. (n=4).

2.3.6 Platelet Aggregation and Adhesion to Combined Agonists Collagen, Adrenaline and TRAP-6 Using the 96-Well Plate Method

These combination studies were performed to mimic more closely the physiological condition of platelets stimulated by multiple factors within the vascular system. However, the combination of either collagen or adrenaline with TRAP-6 did not result in aggregation responses that were any different to those in response to single agonists. In contrast, combination of collagen and adrenaline did result in additive effects on platelet aggregation. For example at 4min, percentage of aggregation by combined 1µg/ml collagen and 10^{-7} M adrenaline was 56±16% compared with 1µg/ml collagen alone, 27±12% or 10^{-7} M adrenaline alone, 22±5%. At 8 min, combinations of 10^{-7} M adrenaline with 0.1, 0.3 and 1 µg/ml collagen enhanced the aggregation to 61±12%, 64±14 and 79±9 respectively as compared with collagen alone (0.1µg/ml, 34±10; 0.3µg/ml, 42±14; 1µg/ml, 57±7) or 10^{-7} M adrenaline, 46±9% (Figure 2.17-2.19). As shown in Figure 2.20, platelet adhesion was increased in combined collagen and adrenaline but not when collagen alone is used. Combination of adrenaline and TRAP-6 or all three agonists did not have additive effects as compared to respective single or dual combination agonists. To further understand this, levels of thromboxane B_2 in the plasma was determined, however no significant difference was found between single and combined agonists (Figure 2.21).

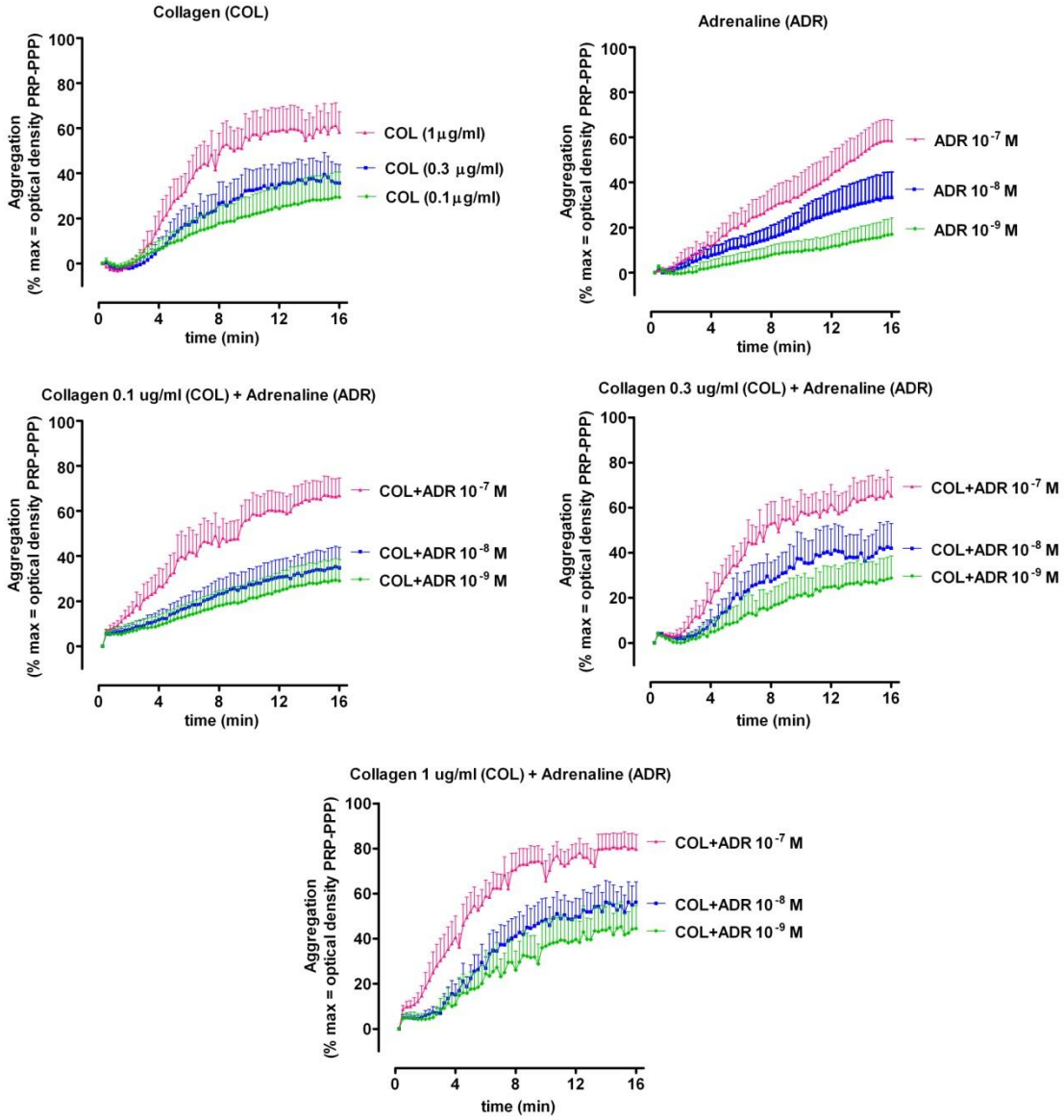


Figure 2.17: *Aggregation traces of single concentrations of collagen or in combination with adrenaline.* Human PRP was stimulated with collagen (0.1, 0.3 and 1µg/ml) and/or adrenaline (10^{-9}, 10^{-8}, 10^{-7}M). Each value represent mean ± SEM (n=8).

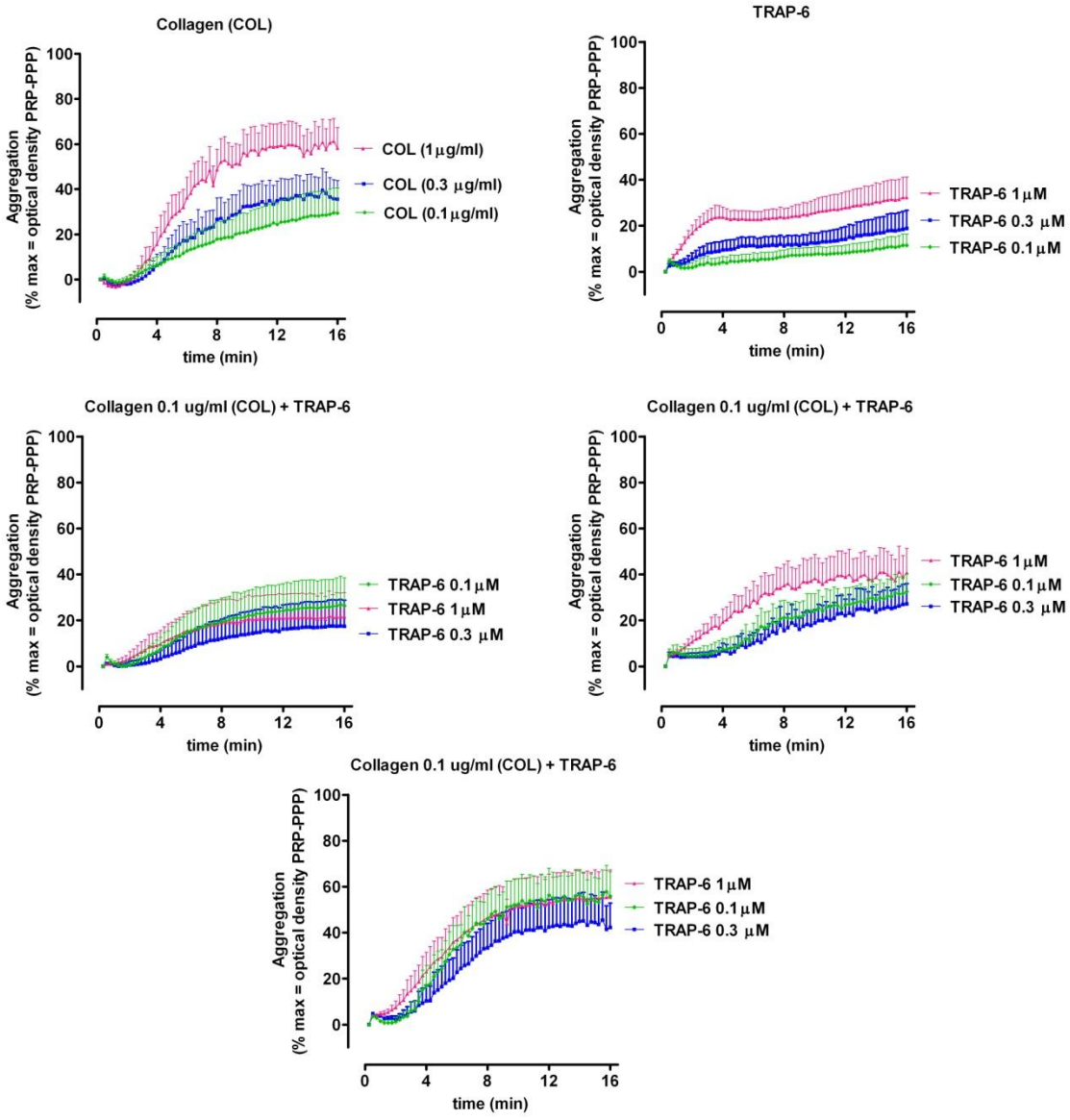


Figure 2.18: *Aggregation traces of collagen alone or in combination with TRAP-6.* Human PRP was stimulated with collagen (0.1, 0.3 and 1µg/ml) and/or TRAP-6 (0.1, 0.3 and 1µM). Each value represent mean ± SEM (n=8).

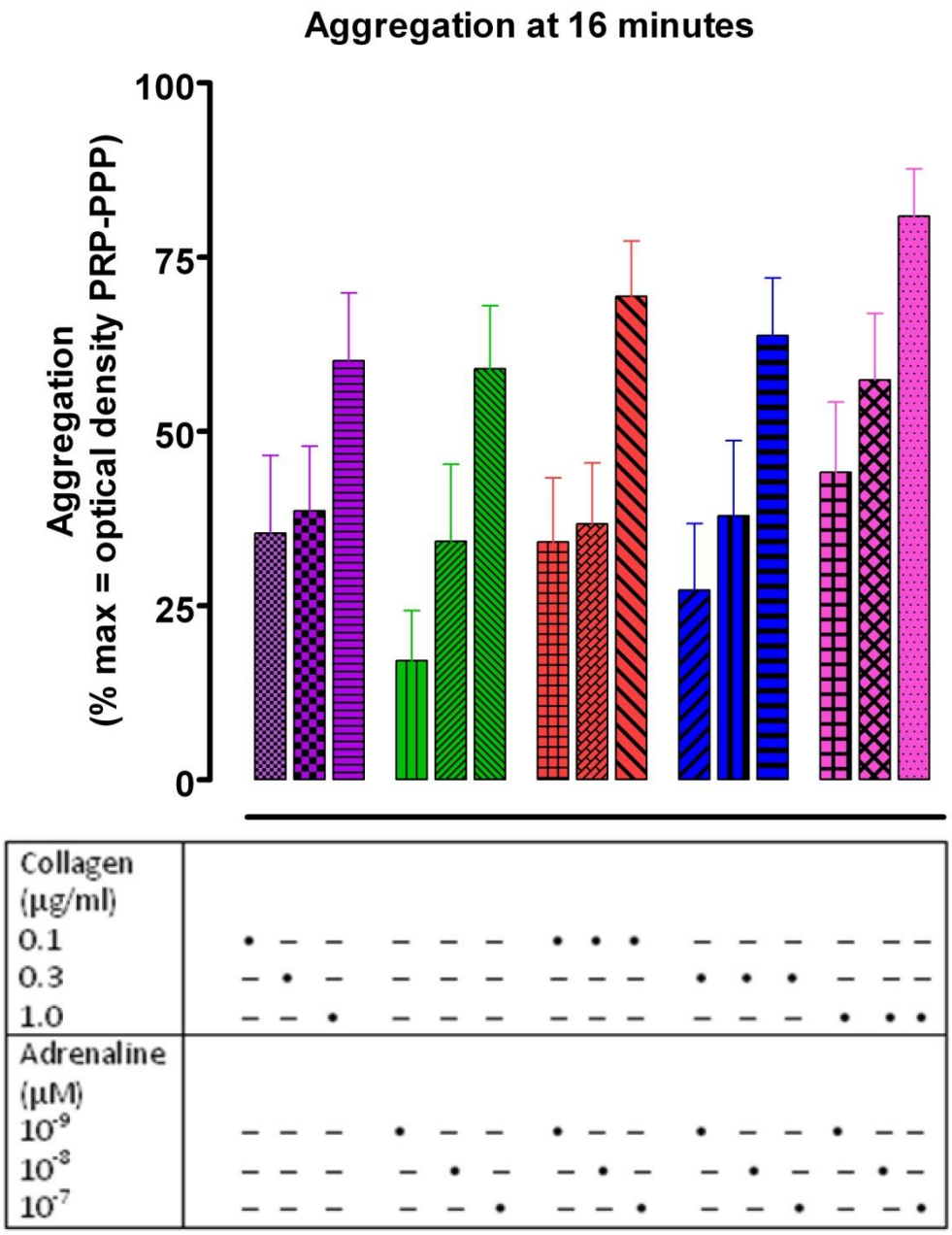


Figure 2.19: *Platelet aggregation at 16 minutes induced by combinations of collagen (0.1, 0.3 and 1µg/ml) and adrenaline (10^{-9}, 10^{-8} and 10^{-9}M) respectively.* Each value represent mean ± SEM (n=8).

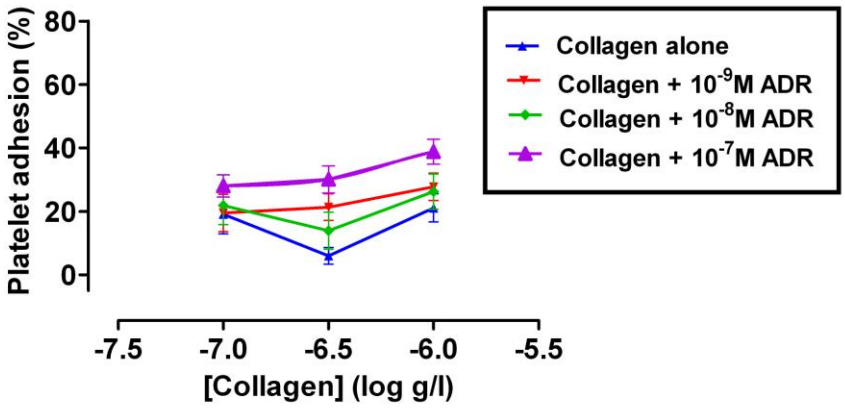


Figure 2.20: *Platelet adhesion induced by combinations of collagen (COL) and/or adrenaline (ADR).* Each value represent mean ± SEM (n=4).

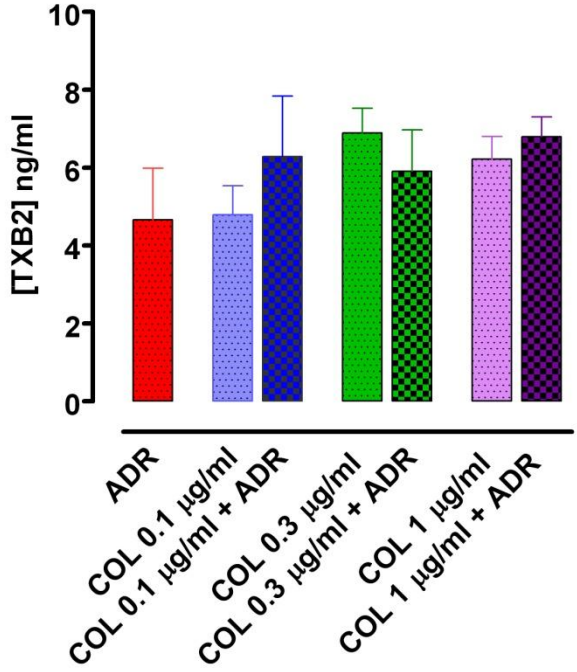


Figure 2.21: *Levels of thromboxane B_2 in PRP following aggregation induced by combinations of collagen (COL) and/or adrenaline (ADR) at 16 minutes.* Each value represent mean ± SEM (n=4).

2.3.7 Inhibition by Aspirin of Platelet Aggregation and Adhesion Induced by Combinations of Collagen and Adrenaline

Combination of collagen 1µg/ml with adrenaline, 0.01µM or 0.1µM, was chosen to test the effects of aspirin on platelet aggregation and adhesion. Combination of collagen with 0.1µM adrenaline significantly increased the platelet aggregation to 76±3% as compared to collagen alone, 61±8%, and adrenaline, 26±5%, respectively (n=8, $p<0.0001$) (Figure 2.23). Aspirin inhibited platelet aggregation induced by collagen plus adrenaline either alone or in combination; e.g. aggregation induced by 1µg/ml collagen plus 0.01µM adrenaline was reduced by 30µM aspirin to 26±7%, as compared to control, 56±7% (n=8; $p<0.0001$). For the combination of collagen and 10^{-7}M adrenaline control aggregation, 76±3%, was reduced to 40±3%, 48±3%, and 54±3%, when treated with aspirin at 30, 100, and 300µM aspirin respectively ($p<0.0001$). Platelet adhesion was also inhibited by aspirin, against both single and combined agonists (Figure 2.24). For instance, platelet adhesion of combined collagen and 0.1µM adrenaline, 37±3%, was decreased by treatment with aspirin; for example, in the presence of 10µM and 30µM aspirin platelet adhesions were 30±2%and 26±2%, respectively.

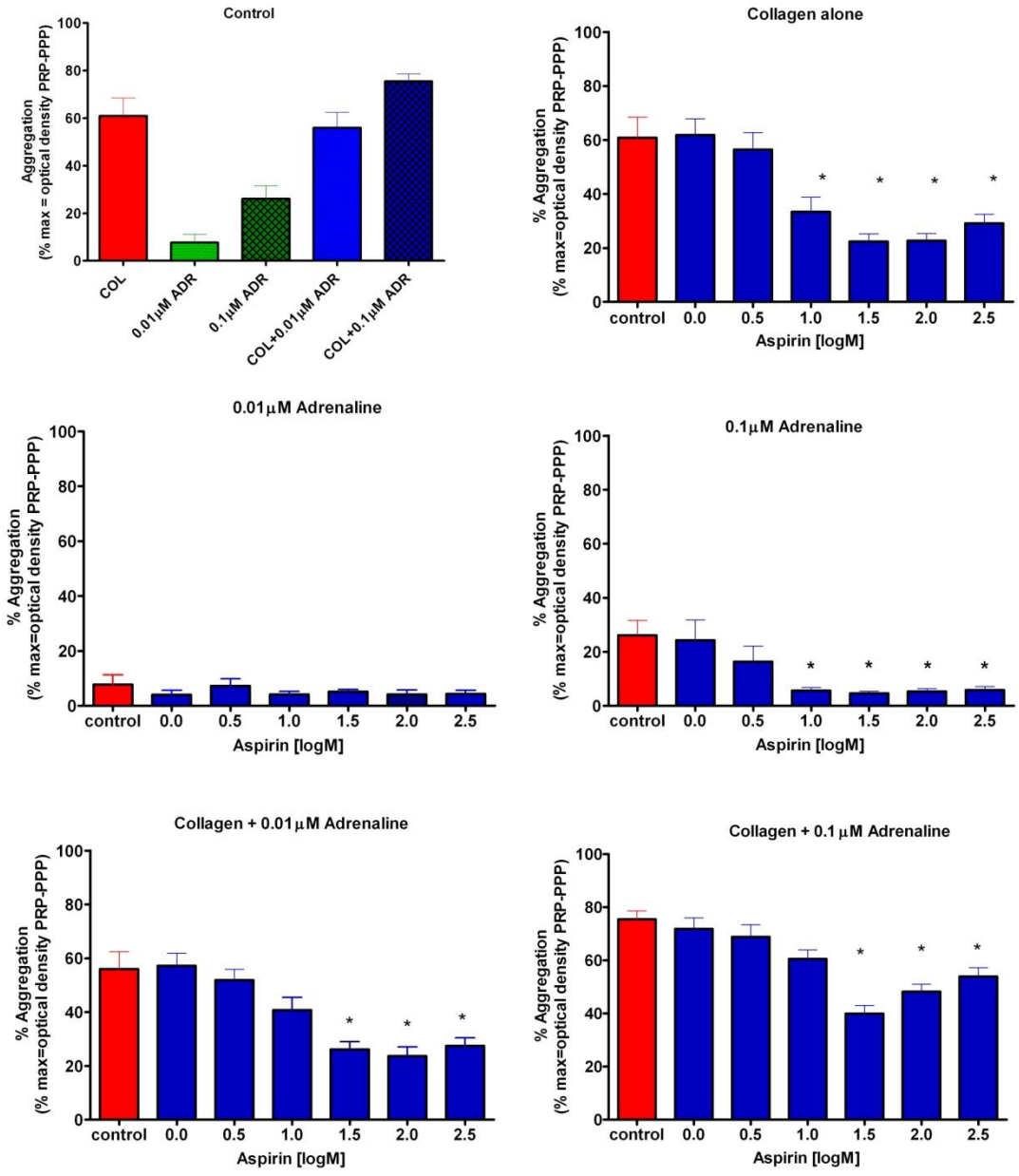


Figure 2.23: *Inhibition by aspirin of platelet aggregation induced by combined agonists.* PRP was incubated with aspirin at various concentrations ranging from 1µM-300µM for 30 minutes before addition of platelet agonists. Each value represent mean ± SEM (n=4). * indicates $p<0.0001$ by one-way ANOVA.

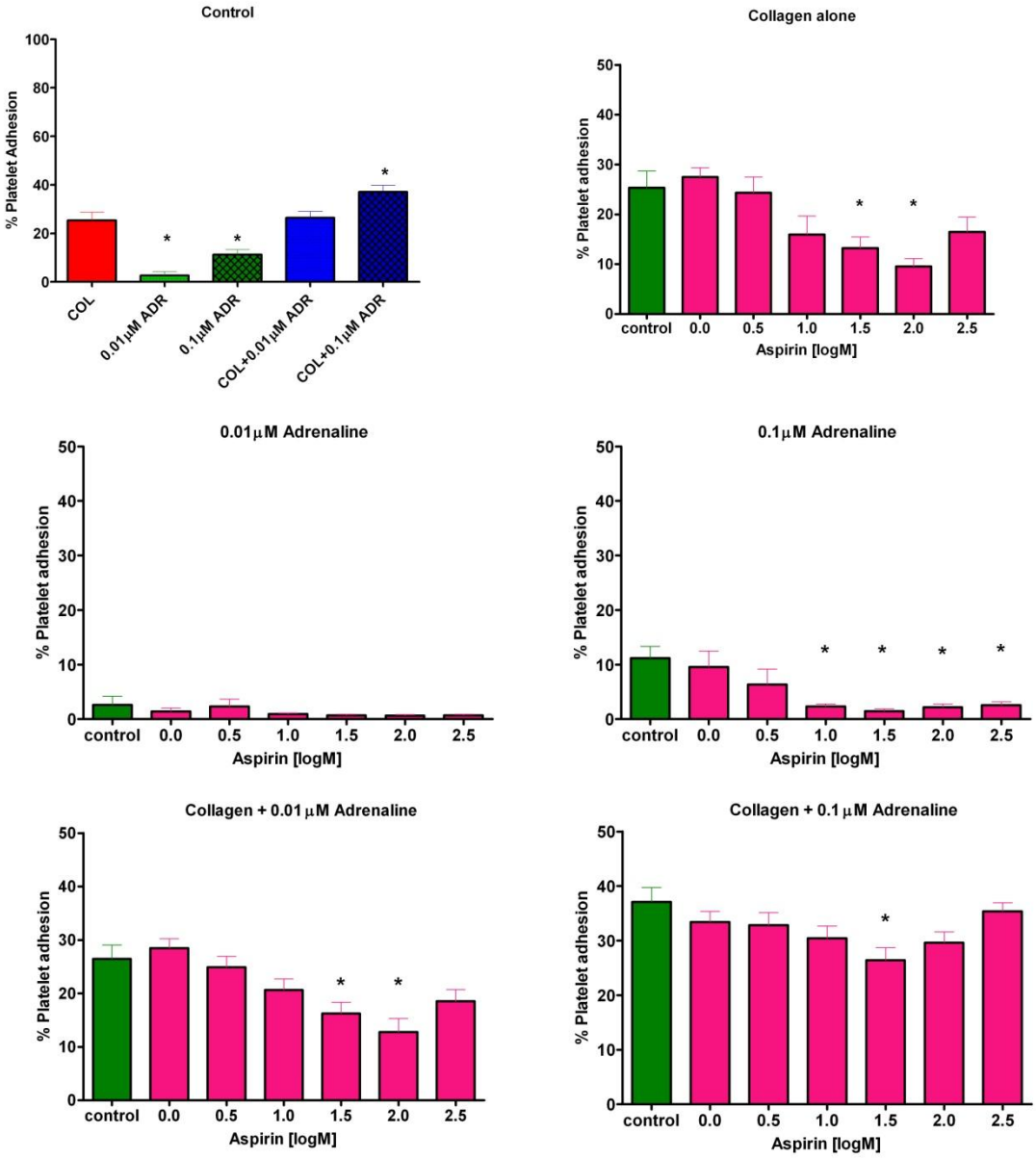


Figure 2.24: *Inhibition by aspirin of platelet adhesion induced by combined agonists.* PRP was incubated with aspirin at various concentrations ranging from 1μM-300μM for 30 minutes before addition of agonists. Each value represent mean ± SEM (n=4). * indicates p<0.0001 by one-way ANOVA.

2.4 Discussion

Platelets are essential for normal haemostasis. For instance, platelets are crucial to arrest bleeding from both the arterial and venous circulations. In pathological conditions, platelets are the major contributor to arterial thrombosis, which often occurs at sites of atherosclerosis within vessel lumens. Because platelets are keys to thrombus formation, the mechanisms underlying platelet adhesion and aggregation are of particular interest to the study of cardiovascular disease.

The aim of this study was to modify an existing method to allow the measurement of platelet aggregation and adhesion in platelet-rich plasma (PRP) using 96-well plates with minimal effort, preparation steps and equipment. Modification of the acid phosphatase assay provided a straightforward and responsive method for the measurement of platelet numbers in platelet suspension after stimulation with platelet agonists (Bellavite *et al.*, 1994). Bellavite *et al.* (1994) showed that this method works for the evaluation of platelet number irrespective of whether the platelets are resting or agonist-stimulated because acid phosphatase activity is not affected by the functional state of the platelet. Platelet quantification using this assay is based on the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol by cytosolic acid phosphatase. Consequently, we can estimate platelet number as acid phosphatase activity is proportional to the platelet number. Furthermore, previous studies have demonstrated the use of acid phosphatase assay to count various adherent and non-adherent cells in a manner which can have higher sensitivity and

reproducibility than cell proliferation assays (Yang *et al.*, 1996). Usefully, platelet adhesion measured by the acid phosphatase method requires no radioactive procedures and produce non-hazardous waste. Modification of the platelet adhesion assay to a 96-well microplate allows larger numbers of samples to be assayed in shorter periods of time and for the comparison of multiple experimental conditions, for example the use of different agonists at various concentrations. Initial study of platelet adhesion in 96-well plate using modified Bellavite method involved the dilution of PRP in dilution buffer or PPP to increase the reproducibility of the assay and sensitivity of yellow *p*-nitropenol detection especially if inhibitory effects of platelet antagonist are used. However, PRP diluted in PPP appreared to give low response when stimulated by agonist as compared to PRP diluted in Dilution Buffer. This may be because of the use of sodium citrate as anticoagulant which effects of chelating extracellular calcium in the plasma is still present in PPP, therefore reducing platelet activation responses by agonist tested in these experiments. Since the use of PRP can produce similar results for platelet adhesion studies as the use of platelet suspensions, platelets in PRP were used throughout the study instead of platelets prepared in dilution buffer or platelet-poor plasma (PPP). Furthermore, platelet count or standardisation of platelet density is not required if using PRP (Eriksson *et al.*, 2005). PRP also provides a physiological milieu in which to test platelet function with the presence of various adhesive plasma compounds and requires less pre-procedure preparation of platelets that can affect platelet function.

The extracellular components that react with platelets include different types of collagen, von Willebrand factor (vWF) and other adhesive proteins such as thrombospondin. Platelet activation also leads to a conformational change of receptors such as GPIIb/IIIa that facilitate fibrinogen binding and platelet aggregation. Even though fibrinogen is not synthesized by vascular wall cells, it must be considered as a potentially relevant thrombogenic substrate as it becomes immobilised onto extracellular matrix at sites of injury. In this study, platelets showed better adherent to fibrinogen and non-coated 96-well microplates than albumin coated plates when activated by ADP. This result is similar to a previous study that reported ADP-stimulated platelet adhesion in a concentration-dependent manner to fibrinogen coated plate, but not to albumin (Bellavite *et al.*, 1994).

Prostacyclin is a potent platelet inhibitor, and vasodilator, that is produced by the COX pathway mainly in the vascular endothelium. Together with TXB_2, prostacyclin plays an important role in an 'agonist-antagonist' relationship in vascular hemostasis (Dogné *et al.*, 2005). Previous study demonstrated that *in vitro* platelet prostacyclin receptor desensitization caused a marked augmentation of platelet-endothelial cell adhesion, thus supports previous evidence showing prostacyclin inhibits platelet adhesion (Darius *et al.*, 1995). By using modified Bellavite's 96-well plate method of platelet adhesion, prostacyclin has been shown to inhibit platelet adhesion, suggesting that this assay can be useful to determine the antiplatelet effects of various agents. Endothelial injury or dysfunction also reduces the production of nitric oxide, which is a key step in atherogenesis and thrombogenesis. Nitrovasodilators, such as SNP, releases nitric oxide

and are used widely in the therapy of cardiovascular artery diseases, most notably for angina (Anfossi *et al.*, 2001). Using this new method of adhesion, the effects of SNP on platelets in PRP diluted in PPP on non-coated plates was examined. SNP inhibited the adhesion of platelets induced by ADP, arachidonic acid, calcium ionophore, adrenaline and collagen. Previous studies have shown that SNP elevates the intraplatelet levels of cGMP and cAMP which synergise to reduce platelet reactivity (Anfossi *et al.*, 2001). Cyclic GMP-independent effects of NO are associated with the inhibition of protein phosphorylation crucial to calcium entry into platelets and inhibition of TXB_2 receptors (Sogo *et al.*, 2000). It was noted that inhibitory actions induced by cyclic nucleotide-elevating substances are mainly mediated by cyclic-nucleotide dependent protein kinases and interfere at multiple sites of the platelet activation signaling cascades, PLC (Ryningen *et al.*, 1998), PKC, and MAPK pathways which affects several steps of cytosolic Ca^{2+} elevation (Russo *et al.*, 2004).

During blood taking, anticoagulant that was used in this study was sodium citrate as it has become the anticoagulant-of choice for platelet testing for many years. Sodium citrate which mechanism of action is chelating extracellular calcium in the blood prevents coagulation as a result of reducing calcium-dependent responses (Bell *et al.*, 1990). In contrast with sodium citrate, heparin prevents coagulation by forming heparin-antithrombin (AT) complex which inactivates coagulation enzymes such as thrombin and factor Xa (Hirsh *et al.*, 2001). Throughout my studies, all blood taking from human used sodium citrate as standard anticoagulant whilst blood taking from mouse involves the use of heparin to prevent blood coagulation. The reasons behind this was because cardiac

puncture during mouse's blood taking increase the possibility of traumatic blood clot, thus the use of heparin reduced the possibility of blood clotting in the syringe. The most commonly used techniques in measuring platelet aggregation is aggregometry, however it has few weak points such as being time-consuming, and requiring relatively large volumes of blood, skilled staff and special equipment (Moran *et al.*, 2006). In this study, platelet aggregation was adapted to a 96-well plate format which could be read in a normal microplate reader that has shaking properties. The shaking effects applied during the measurement of aggregation imitate the circulating blood platelets that frequently collide with each other or blood vessels. The optimum wavelength with higher sensitivity for aggregation is the shortest one available on the microplate readers, normally 405nm (Bednar *et al.*, 1995). However, due to the yellow colour of plasma which produces a high background signal, we used a much longer wavelength, 595nm. To standardise the method of platelet aggregation, PRP was used as the setting equivalent to 0% aggregation, whilst 100% of aggregation was taken being the maximal transparent reading to PPP. Combination of platelet aggregation and adhesion assay in this 96-well plate method generates more data of platelet function in response with various agonists in a shorter period of time as compared with traditional Born aggregometry. Concern about pipetting PRP into agonist-well at the same time so that the onset of platelet activation is not varies from one well to another was overcome by the use of automated multichannel pipettes. This will not only reduce time taken to add PRP into 96-wells, but will decrease the possibility of bubble formation that can interrupt absorbance reading. In addition, the use

of microplate reader with 12-channel optical system that read the entire 96-well plate at the same time adds to the reproducibility of this assay.

To further maximise the advantages of using 96-well plate format, half-area 96-well plates were tested for human and mouse platelet aggregation and adhesion. This method is very useful because it requires less sample volumes, which could be particularly useful for blood samples obtained from mice or, for instance, human newborns. Furthermore, this new method allows the measurements of platelet aggregation under the influences of various platelet agonists at the same time, thus providing more data in a very short time compared with traditional aggregometry. In this study, increases in human platelet aggregation and adhesion in response to ADP, adrenaline, collagen, TRAP-6, ristocetin, U46619 (thromboxane analogue) and arachidonic acid were seen. Similar observations were made in mouse platelets. As thrombin cannot be used in PRP as an aggregating agent because it stimulates clotting, TRAP-6 was used as it mimics the strong aggregating effect of thrombin. However, mouse platelets did not response to TRAP-6, or to thrombin at the concentrations tested in this study. These may be related to differences in the expression of thrombin receptors, protease-activated receptors (PAR) in human and mouse platelets. Human platelets express PAR-1, a high-affinity receptor that is activated at low concentrations of thrombi and PAR-4, a low affinity receptor that mediates thrombin signalling at higher concentrations (Ofosu, 2003), whereas mouse platelets express PAR-3 and PAR-4. In mouse platelets, PAR-3 serves as a co-receptor for PAR-4, wherein thrombin binds to PAR-3 and PAR-4, and cleaves the amino terminus of PAR-4

(Mao *et al.*, 2008). Therefore, because TRAP-6 activates platelet through the PAR-1 receptor mouse platelets are not activated by TRAP-6. Although thrombin activates mouse platelets, the thrombin that was used in this study may not fully stimulate the thrombin receptors of mouse platelets, thus the weak activation may not have been sufficient to stimulate platelet aggregation.

Peroxisome proliferator-activated receptor- (PPAR-γ) plays a crucial role in immune function by suppressing inflammation and attenuating macrophage/monocyte formation of proinflammatory cytokines. It had been thought that PPAR-γ is expressed only in nucleated cells since it is known as a transcription factor mainly located in the nucleus, however, recent studies have shown that PPAR-γ is also present in the anucleated platelet (Akbiyik *et al.*, 2004). PPAR-γ is important for regulating gene expression in metabolism, insulin reactivity, and adipocyte differentiation. Thiazolidinediones (TZDs) such as rosiglitazone are insulin-sensitising agents which exert their effects through PPAR-γ. It has been reported that treatment with TZDs is linked with significant improvements in surrogate markers of cardiovascular disease, including lipid profile, blood pressure and markers of inflammation and oxidative stress (Khanolkar *et al.*, 2008). In addition to this, previous studies have shown that platelets express functional PPAR-γ (Akbiyik *et al.*, 2004). Despite the largely unknown function of PPAR-γ in platelets, clinical data indicate that rosiglitazone reduces platelet activation in non-diabetic patients with coronary artery disease and reduces post-coronary stent restenosis rates in patients with Type-II Diabetes Mellitus (T2DM). This is supported by recent studies which have demonstrated that

rosiglitazone attenuates platelet activation as measured by reduced platelet aggregation and sCD40L in patients with T2DM (Khanolkar *et al.*, 2008). Previous evidence of the presence of all PPAR sub-family in non-nucleated platelets resulted in more research has been done to study the role of PPARs in platelets (Bishop-Bailey, 2010). For example, PPARβ has been shown to have synergistic antiplatelet effects with NO, suggesting PPARs activation is crucial in inhibition of platelet function by prostacyclin (Ali *et al.*, 2005). Another family of PPARs, PPARγ also showed a functional role in inhibition of platelet function in vivo (Li *et al.*, 2005). In this study, pioglitazone, an inhibitor of PPARγ reduced arterial thrombus formation in rats fed with pioglitazone as compared with normal chow fed rats. Although rosiglitazone at tested concentration failed to show any inhibitory effects on platelet aggregation and adhesion in this study, previous investigation showed that activation of PPARα and PPARγ is important in the antithrombotic effects of statins and fibrates, thus suggesting that PPARs is a mediator for platelet inhibition (Ali *et al.*, 2009).

The platelet responses to different platelet agonists can be enhanced when they are present in low concentrations, or are added together or in sequence to PRP (Steen *et al.*, 1988). Synergistic effects of thrombin and adrenaline have been demonstrated in platelet aggregation, granule secretion and mobilization of cytoplasmic Ca^{2+}. In the 96-well plate format additive effects of combinations of low concentrations of collagen and adrenaline were found on platelet aggregation. However, combination of either collagen or adrenaline with TRAP-6 did not produce any additive effects on platelets. This finding

could be indicative since low concentrations of several agonists may mimic the conditions under which thrombosis occurs *in vivo*. Furthermore, this shows that the 96-well format could be used as cheaper alternative of using combined platelet agonists as employed in machines such as the PFA-100. In the PFA-100, whole blood is transferred into standard cartridges that contain membranes coated with collagen and either ADP or adrenaline. The time necessary to occlude the microscopic aperture in the cartridge is measured and reported as closure time. PFA-100 closure times are known to be influenced by several factors such as platelet count, hematocrit, platelet activity and von Willebrand factor. Therefore, combined agonists 96-well plate method may offer another approach to platelet function testing that is less influenced by the factors that can confound the PFA-100.

The use of combinations of platelet agonists in the 96-well plate method of platelet aggregation and adhesion was further tested for utility in evaluating the effects of platelet inhibitors by examining the effects of aspirin. Inhibition of platelet function by aspirin is dependent upon the inhibition of platelet COX-1 that catalyzes the conversion of arachidonic acid to PGH_2 and then via thromboxane synthase to TXA_2 (Howard *et al.*, 2004). TXA_2 is a strong platelet agonist that causes platelet aggregation, vasoconstriction and smooth muscle proliferation (Hermann *et al.*, 2006b). Long term aspirin use attenuates the risks or myocardial infarction, stroke, and vascular related deaths in cardiovascular disease patients. Unlike other NSAIDs, aspirin inhibiting effects is irreversible that involves the acetylation of the serine domain located in the active site of

COX (Takahashi *et al.*, 2008), thereby blocking the access of arachidonic acid to catalytic site of COX-1 (Campbell *et al.*, 2007). Platelets are anucleated, and thus are incapable of protein synthesis to repair COX-1 inhibition by aspirin. The prolonged effects of aspirin are overcome by platelet turnover, in which nearly 10% of the platelet population is replaced with new platelets containing functioning COX-1 each day (Burch *et al.*, 1978). This means following termination of aspirin treatment, COX-1 platelet activity is fully restored after around 10 days. However, only small doses of aspirin, as low as 40mg daily, are needed in order to maintain the antiplatelet effects (Zimmermann *et al.*, 2008).

The results presented here demonstrate that the 96-well modified light transmission aggregation assay using low concentrations of agonists in combination is useful for the detection of inhibition of the platelet COX-1 dependent pathway of aggregation by aspirin, as our group has also reported for individual agonists (Armstrong *et al.*, 2008).

VerifyNow™ Aspirin is a point-of-care platelet aggregation test with special cartridges designed to detect aspirin resistance. Nielson and colleagues have studied the effects of aspirin determined by the VerifyNow™ Aspirin System and traditional light transmission aggregometry (LTA) in healthy volunteer and patients with stable CAD (Nielsen *et al.*, 2008). Despite LTA being a labour-intensive and time-consuming procedure, in contrast with VerifyNow™ Aspirin System, this study shows that LTA detected higher aggregation levels in patients compared to healthy volunteers. In addition, several subjects with 'aspirin resistance' were found by LTA but not by VerifyNow™ Aspirin System (Nielsen *et*

al., 2008). Therefore, this combined agonist's setup in 96-well plates that uses minimal concentration of platelet agonists may provide another alternative method to determine the effects of aspirin in patients with sensitivities and advantages similar to those of LTA. Furthermore, a previous study has shown the uses of combined agonists to detect the inhibitory effects of aspirin against the release of 5HT platelets in whole blood (May *et al.*, 1997). In this study, use of single agonists ADP, adrenaline or PAF did not produce platelet 5HT release, but combination of any two or all of the agonists caused release of 5HT that was inhibited by aspirin. Thus, a combined agonist method may provide a better understanding of the additive or synergistic effects of platelet agonists that could be more useful in monitoring the effectiveness and influences of aspirin therapy.

Overall, the use of 96-well plate assays could have great utility as a common approach to determine platelet function, notably aggregation, adhesion, and release reactions. Even though this method uses PRP that has to be processed from whole blood, it is highly repeatable, cost and time-effective, and particularly useful for testing large numbers of samples or assay conditions. It could well be useful in both the clinical setting and also for basic research into platelet function.

CHAPTER THREE:

INVESTIGATION OF THE EFFECTS OF INHIBITION OF COX-1 AND COX-2 ON PLATELET FUNCTION

3.1 Introduction

Arachidonic acid metabolism and the formation of eicosanoids is central to the regulation of the cardiovascular system through a variety of mechanisms (Linton *et al.*, 2008). The syntheses of PGs are cell specific, for instance TXA_2 is produced by platelets via COX-1, but can also be produced by macrophages via COX-1 and COX-2 (Grosser *et al.*, 2006). Platelet TXA_2 is an important stimulator of platelet aggregation and the platelet inhibitory effects of aspirin that are beneficial for protection against thrombotic events are explained by the blocking of platelet COX-1 pathway and TXA_2 production (Cipollone *et al.*, 2008; Hennekens *et al.*, 1997). COX-2 inhibitor was developed with the objective of reducing the gastrointestinal toxicity associated with the use of NSAIDs and linked to inhibition of gastrointestinal COX-1 (FitzGerald *et al.*, 2001). However, more recently it has become apparent that inhibition of COX-2 could well be associated with an increased risk of thrombotic events.

Thus, the primary objective of this study was to determine the effects of inhibition of COX-1 and COX-2 on platelet function using 96-well plate aggregometry in both *in vitro* and *ex vivo* assays with healthy volunteers, and to investigate changes in blood flow within the forearm caused by reactive hyperaemia in the absence and presence of diclofenac.

3.2 Methodology

3.2.1 Effects of Diclofenac and Parecoxib in vitro

PRP was prepared from whole blood withdrawn from healthy donors as described previously. Diclofenac sodium (Sigma) and parecoxib sodium (Dynastat® 40mg) were used in this study. PRP was incubated for 30 minutes at 37^0C with diclofenac, parecoxib or vehicle before the addition of platelet agonists. Determinations of platelet aggregation and adhesion were made in 96-well plates as described in Chapter 3. Data (n=4) were calculated and analysed using GraphPad Prism 4.0 (GraphPad Software, CA, USA).

3.2.2 Effect of Intravenous Diclofenac on Platelet Function, Prostanoid Production and Forearm Blood Flow in Healthy Adult Volunteers

3.2.1 Study Population and Study Design

8 healthy male individuals $\geq$ 18 years and $\leq$ 40 years were included in the study. Exclusion criteria were smoking or receiving any healthcare treatment, especially with aspirin or any non-steroidal anti-inflammatory drugs (NSAIDs) or other drugs known to affect platelet function (refer lists of inclusion and exclusion criteria below). Ethical approval was obtained from the NHS St. Thomas's Hospital Research Ethics Committee reference 06/

Q0702/150 and conducted according to the Declaration of Helsinki. All volunteers were informed briefly about the clinical trial and were required to sign an informed consent prior of setting a start date. The trial was being conducted with the notification of volunteers' GPs. Study design for this trial was performed as one-centre, double blind, placebo-controlled crossover mechanisms of action study in which all healthy male volunteers received treatment of diclofenac sodium or normal saline during two scheduled visit (Figure 2.1).

3.2.2 Inclusion Criteria

Volunteers met the following criteria to be included in this study:

1) Caucasian, male and aged between 18 and 40 years of age.
2) Free of significant abnormal findings as determined by medical history, screening physical examination, haematology, biochemistry, urinalysis (including specific gravity), and vital signs (sitting blood pressure, sitting pulse rate, sitting respiratory rate and body temperature) within 2 weeks of commencement of the study.
3) Normal fasting lipid profile.
4) Non-smoking (due to vasoactive and pro-aggregatory effects of nicotine).
5) Clear venous access in upper limbs.
6) BMI between 18 and 30.
7) No history or signs of drug abuse (including alcohol), licit or elicit.

8) Agrees not to use any medications (prescribed or over-the-counter including herbal remedies) judged to be clinically significant by the Principle Investigator during the 4 weeks preceding the study, and during the course of the study.
9) Able to understand and sign the written Informed Consent Form.
10) Able and willing to follow the Protocol requirements.

3.2.3 Exclusion Criteria

Volunteer were not included in this study if any of the criteria below applied:

1) Females, due to the confounding effects of the menstrual cycle on circulatory behaviour.
2) Smoking and tobacco consumption, due to confounding effects on circulatory behaviour.
3) Any significant history of allergy and/or sensitivity to any of the contents of either the study drugs or to any other NSAIDs.
4) Any evidence or history of organ dysfunction, or any clinically significant deviation from normal in the physical or clinical determinations.
5) History of disorders of the gastrointestinal, hepatic, renal, cardiovascular, endocrine (including diabetes), neurological (including epilepsy, migraine headaches, depression and convulsions), metabolic, psychiatric, haematological (especially anaemia and coagulation disorders) or systemic disease judged to be clinically significant).

6) Asthma.

7) A pulse rate of less than 50 beats/minute, a sitting systolic blood pressure >160 or <80 mmHg and/or a sitting diastolic pressure of >100 or <60 mmHg.

8) Any significant illness during the 4 weeks preceding the screening period of the study.

9) Any contraindication to blood sampling.

10) Positive urine drug screen or indication.

11) Alcohol consumption greater than community norms (for example more than 21 standard drinks per week for males).

12) Participation in any clinical study during the weeks preceeding the dosing period of the study.

13) Donation of blood during the 8 weeks preceeding the screening period of the study or during the investigation.

14) Concerns regarding the subject's participation in the study are raised by their GPs.

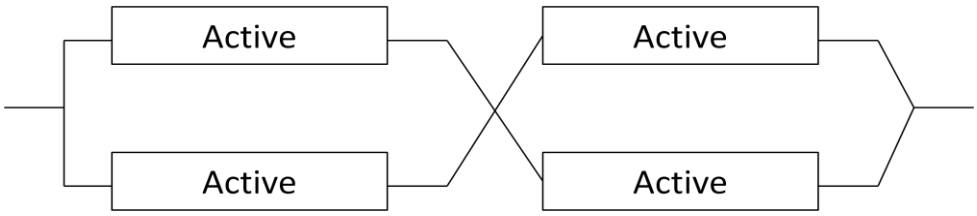


Figure 3.1: *Study design for the evaluation of intravenous diclofenac on platelet function and post-hyperaemia prostanoid production in healthy adult volunteer.*

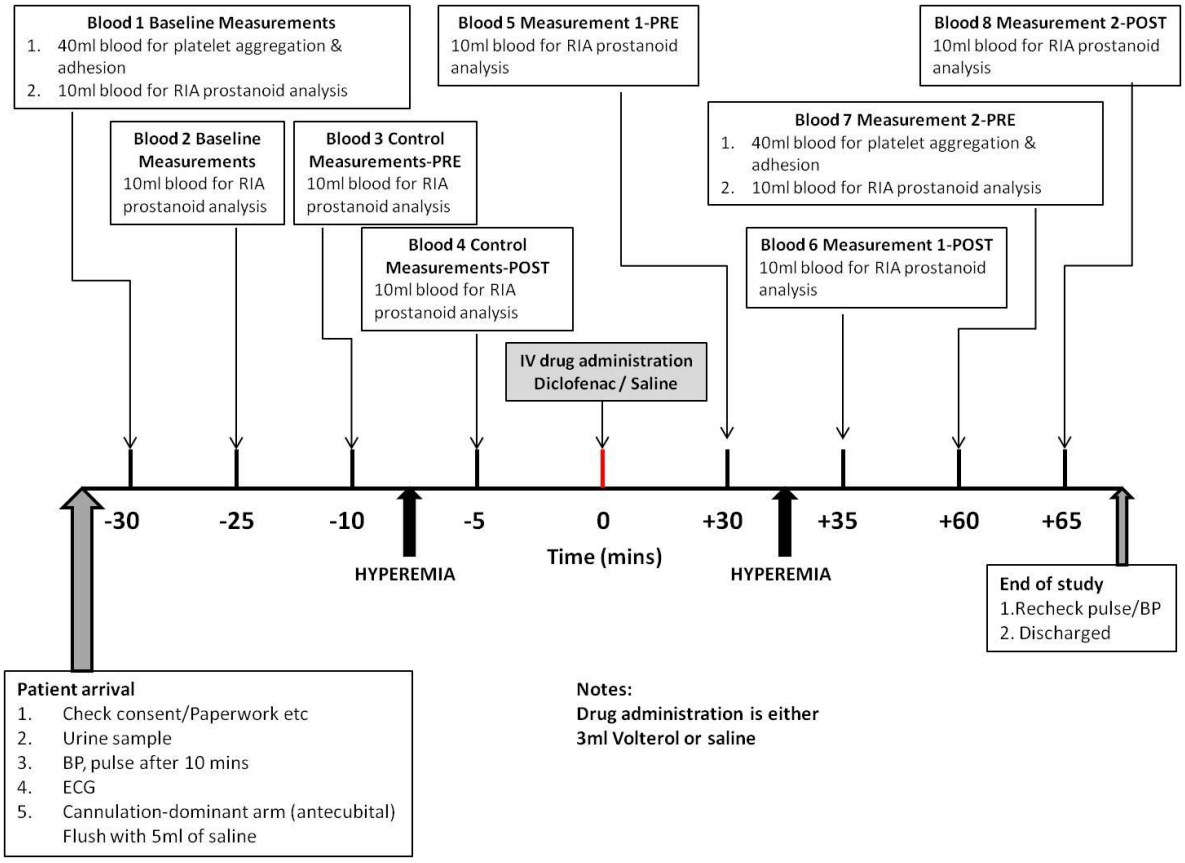


Figure 3.2: *Diagram above shows detail of visit schedule which includes blood taking and drug administration time.* There were two visits for each participants following randomisation visit and participants will be administered with diclofenac or control saline in each visit. There was 4 weeks interval between visits to allow wash out period.

3.2.4 Drug Administration

The active agent, Voltarol®Novartis (25mg/ml diclofenac sodium) was given as intravenous administration with total dose of 75mg/3ml into the arm vein of the dominant arm followed by 10 ml of normal saline flush. 3 ml 0.9% physiological saline was given intravenously for placebo control. During crossover a period of at least 4 weeks was left to allow full drug washout and both blood cell and platelet replacement (Figure 3.2).

3.2.5 Platelet Study

For the purposes of the platelet study, 52 ml of venous blood was taken from each participant before and after administration of treatment. The first 2 ml of blood for each collection was discarded as this represents residual blood and saline from within cannula. The same amount of normal saline was returned via the cannula for blood replacement. Study of platelet reactivity was conducted as platelet aggregation followed by platelet adhesion, as described in Chapter 3. Quantification of platelet adhesion was made immediately after platelet aggregation.

3.2.6 Platelet Function Analyser-100 System (PFA-100)

Blood samples from all subjects were taken to measure platelet function by use of the Platelet Function Analyser (PFA-100®, Dade Behring). In this instrument, citrated whole blood is aspirated at high shear rates (5000-6000 s-1) through a glass capillary (diameter 200 μm) into a membrane pore (diameter 150 μm). The membrane is coated with 2 μg of type I collagen and 10 μg adrenaline or 50 μg ADP. Platelet function is measured as a function of the time (closure time/ CT) that platelets take to occlude an aperture in a coated membrane; both types of cartridges were used in this study, collagen/adrenaline (CEPI) and collagen/ADP. Cartridges were prewarmed to room temperature before loaded into the device.

3.2.7 Postocclusive Forearm Skin Reactive Hyperaemia

Participants were in a supine position with arms and hands kept stationary. The uncovered right arm was supported comfortably with cushion and was slightly lifted above heart level. Forearm blood flow was then occluded using a pneumatic pressure cuff (Accoson, England) placed on the non-dominant upper arm about 1-2cm above the antecubital crease following inflation to a suprasystolic pressure (systolic blood pressure + 10 mmHg) for 3 minutes. Reactive hyperaemia was produced by the rapid release of the cuff to re-institute blood flow. Blood samples were taken before and after each forearm occlusion for baseline and after drug administration.

3.2.8 Prostanoid Production by Radioimmunoassay

Serial 10 ml blood samples were collected for measurement around baseline hyperaemia (pre-intravenous injection of drug) and around hyperaemia after intravenous drug administration. One portion of blood was centrifuged to prepare plasma and stored for future analysis of thromboxane B_2, a stable metabolite of thromboxane A_2. Another portion of blood was incubated with 500 µM of calcium ionophore or 1mg/ml LPS for 30 min to activate the COX pathway and prostanoid production by platelets. Samples were centrifuged to obtain plasma and stored for analysis of TXB_2 and PGE_2 by radioimmunoassay, as described previously.

3.3 Results

3.3.1 Part One: Effects of Diclofenac and Parecoxib in Vitro

3.3.1.1 Inhibition by Diclofenac (1mM) of Platelet Activation by Various Agonists

AA-induced effects on platelet aggregation and adhesion were inhibited by diclofenac. For instance, aggregations induced by 0.3, 1.0 and 1.6mM AA were decreased to 4±1%, 1±4% and 0% from 47±23%, 54±20% and 79±16%. Adhesions stimulated by same concentration of AA were also inhibited from 22±9%, 22±9% and 27±4% to only 1% for all three concentrations. Collagen and adrenaline were also strongly inhibited by diclofenac. Stimulation by collagen at 3, 10 and 30µg/ml induced aggregations of 55±9%, 88±6% and 91±2% which were decreased to 19±4%, 58±4% and 66±4%, respectively, by diclofenac. Diclofenac also reduced aggregation induced by adrenaline at 1, 10 and 100µM to 22±9%, 34±13% and 37±12% as compared to the controls, 54±15%, 66±15% and 72±10%, respectively. Diclofenac also significantly inhibited platelet adhesion induced by collagen and adrenaline. For example, at 3 and 10µM collagen, platelet adhesion was decreased to 11±3% and 32±3% as compared to 25±4% and 44±4% in control, respectively. Adhesion induced by 10 and 100µM adrenaline was also decreased from 24±6% and 29±4% to 15±5% and 17±5% following incubation with diclofenac. ADP, TRAP-6 and ristocetin-stimulated platelets also showed significantly decreased platelet aggregations after

treatment with diclofenac. However, only ADP-stimulated platelet adhesion was inhibited by diclofenac. In addition, platelet aggregation in response to lower concentrations of TRAP-6 was more greatly inhibited by diclofenac. In contrast to the other agonists, U46619-stimulated platelet activation was not affected by diclofenac (Figure 3.3-3.7).

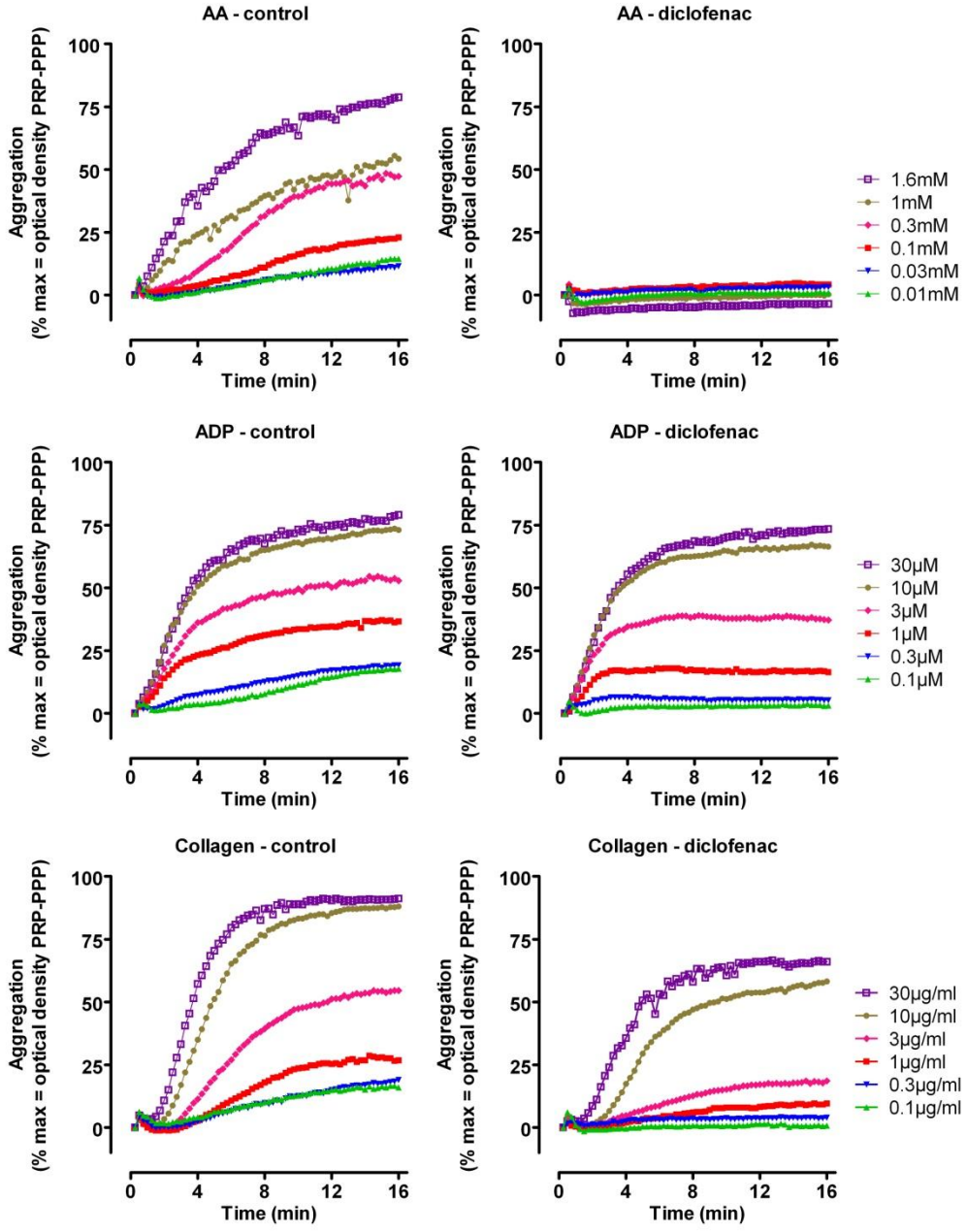


Figure 3.3: *Aggregometery traces showing effects of diclofenac on platelet aggregation induced by AA, ADP and collagen.* PRP was incubated for 30 min, 37^{0}C with 1mM diclofenac before agonist-stimulated platelet aggregation was determined in 96-well plates. Each data point represents mean ± S.E.M. (n=4-5).

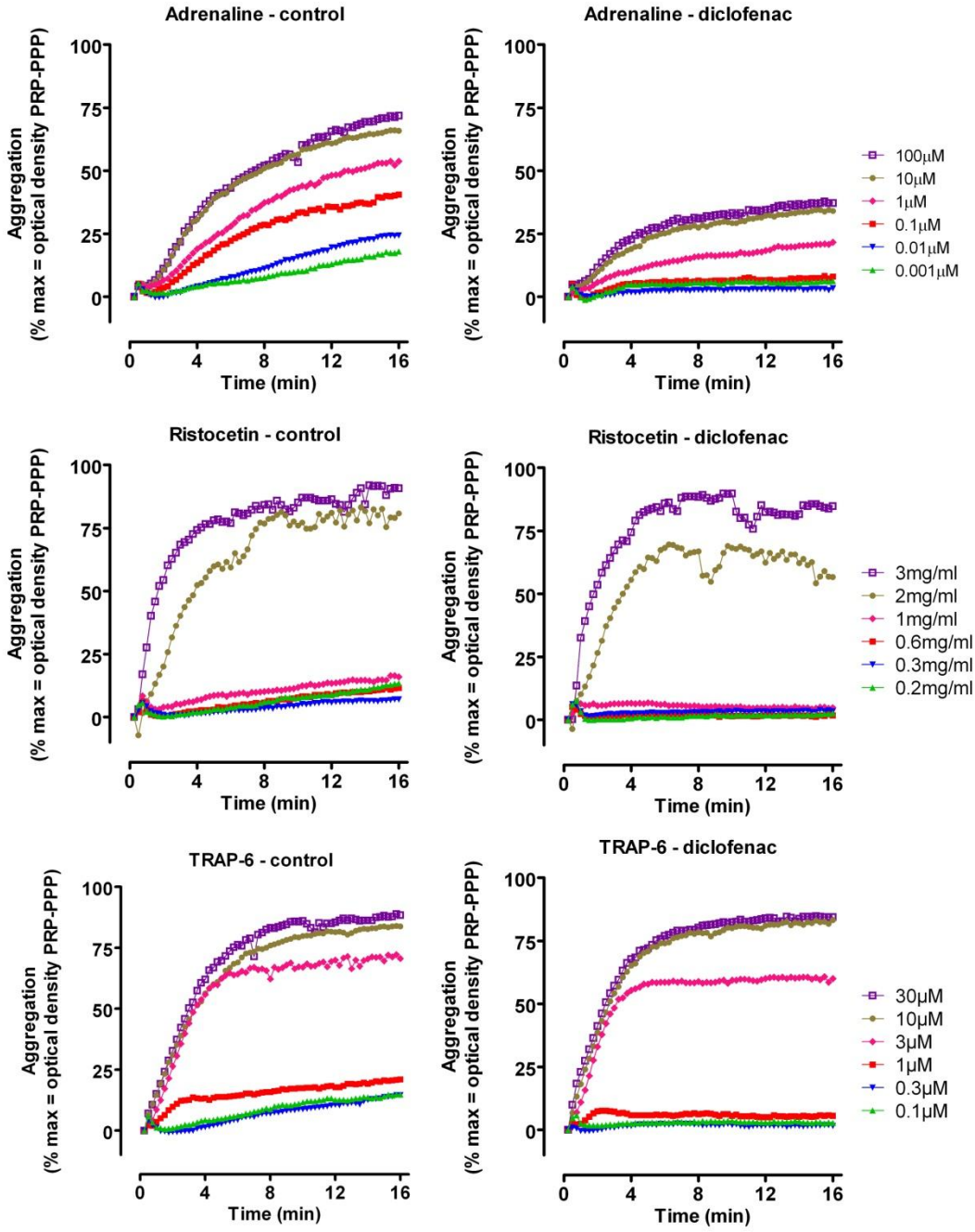


Figure 3.4: *Aggregometry traces showing effects of diclofenac on platelet aggregation induced by adrenaline, ristocetin and TRAP-6.* PRP was incubated for 30 min, 37^{0}C with 1mM diclofenac before agonist-stimulated platelet aggregation was determined in 96-well plates. Each data point represents mean ± S.E.M. (n=5).

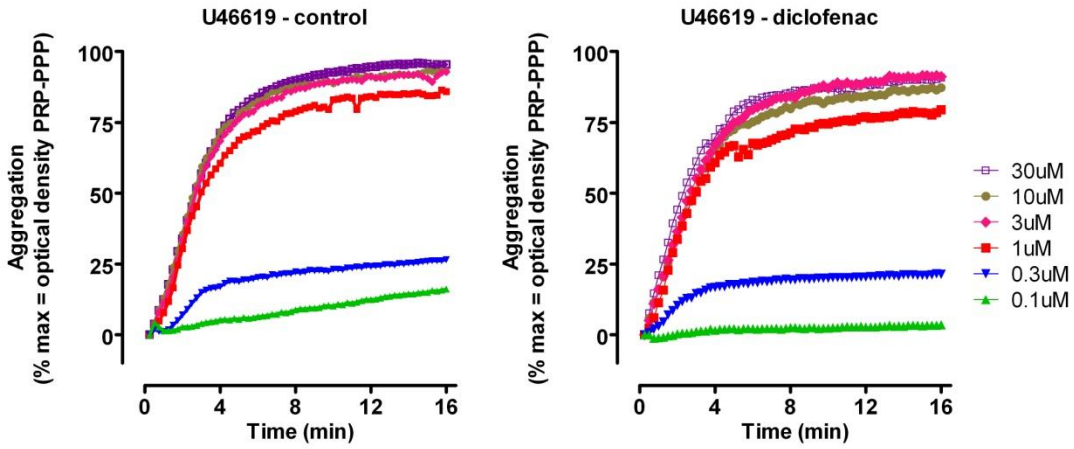


Figure 3.5: *Aggregation traces of the effects of diclofenac on platelet aggregation induced by U46619.* PRP was incubated for 30 min, 37^0C with 1mM diclofenac before agonist-stimulated platelet aggregation was determined in 96-well plates. Each data point represents mean ± S.E.M. (n=5).

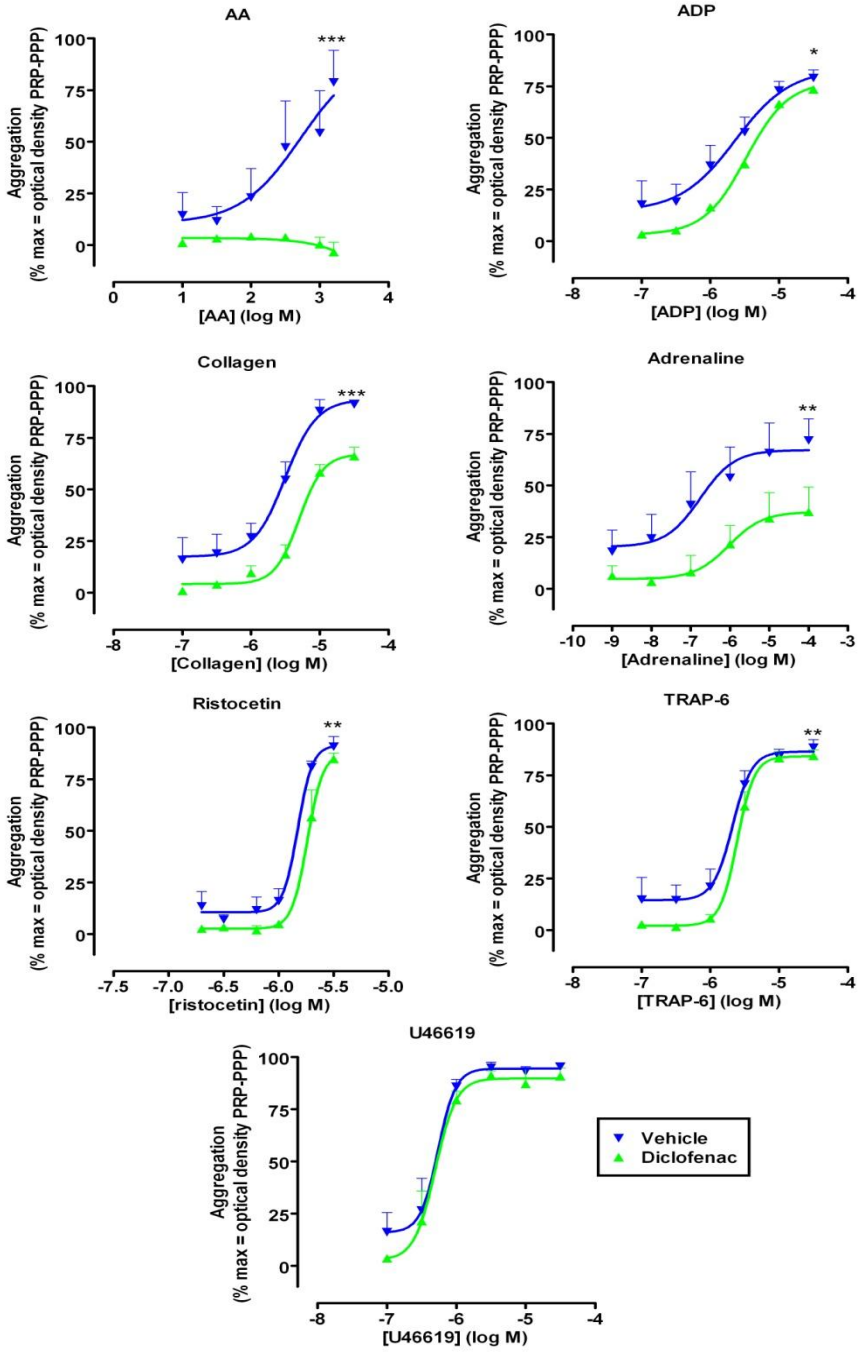


Figure 3.6: *Inhibitory effects of diclofenac on platelet aggregation stimulated by various agonists.* PRP was incubated with 1mM diclofenac before agonist-stimulated platelet aggregation was determined in 96-well plate. Each data point represents mean ± S.E.M. (n=4-5). * = P<0.05; ** = P<0.01; *** = P<0.001 determined by two-way ANOVA.

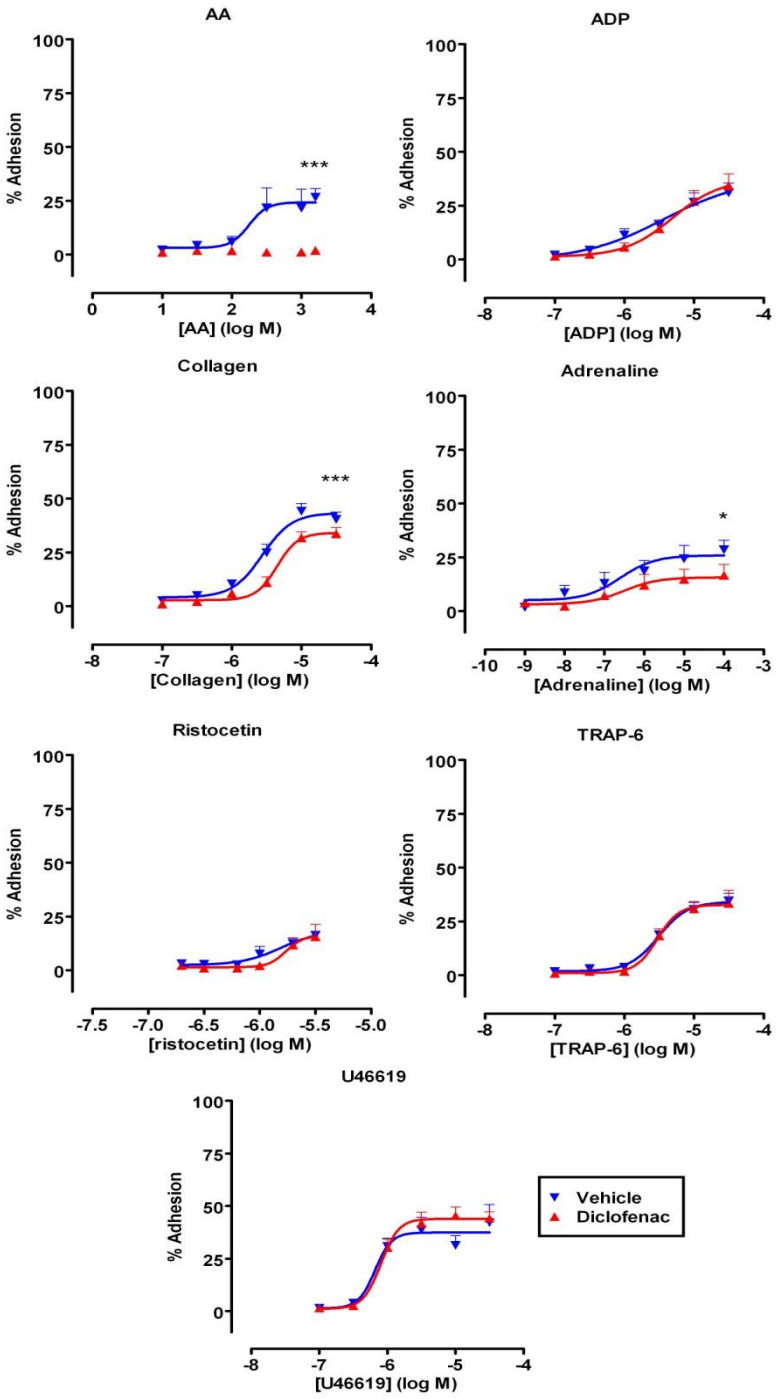


Figure 3.7: *Inhibitory effects of diclofenac on platelet adhesion stimulated by various agonists.* Each data point represents mean ± S.E.M. (n=4-5). * = P<0.05; and *** = P<0.001 determined by two-way ANOVA.

3.3.1.2 Concentration-Dependent Inhibition by Diclofenac of AA-Stimulated Platelet Responses

To determine the concentration-dependent effects of diclofenac the effects of diclofenac 0.001-100µM against the stimulatory effects of AA were measured as shown in Figure 3.8. The concentration-dependent inhibitory effects of diclofenac can be seen clearly against aggregation induced by both 1.0 and 1.6mM AA, and platelet adhesion induced by both 0.3 and 1mM of AA. For instance, aggregation induced by 1.0mM AA was 63±12%, which decreased to 57±13%, 35±15% and 5±2% (p<0.0001) in the presence of 0.001, 0.01 and 0.1µM diclofenac. For adhesion induced by 1.0mM AA, the control response was 29±7% which was decreased to 27±7%, 12±5% and 3±1% (p<0.0001) in the presence of the same concentrations of diclofenac.

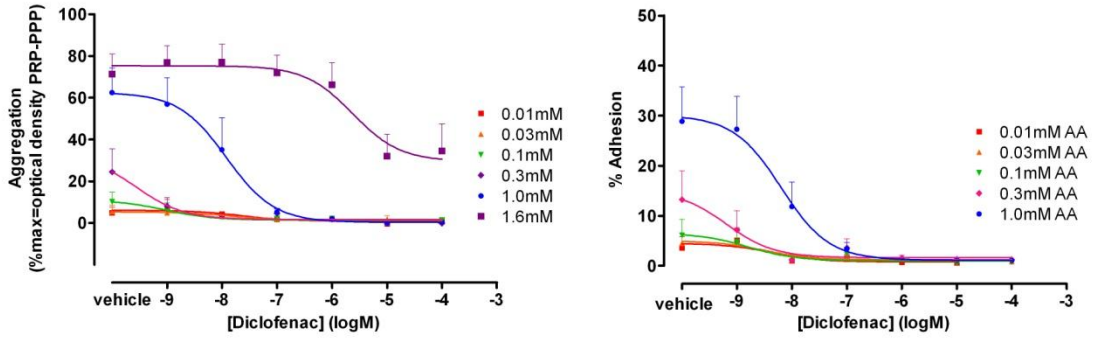


Figure 3.8: *Diclofenac inhibits platelet aggregation stimulated by arachidonic acid (AA) in a concentration-dependent manner.* PRP was incubated with diclofenac 1nM-0.1mM for 30 min, 37^{0}C before determination of platelet aggregation and adhesion induced by various concentrations of AA. Each data point represents mean ± S.E.M., n=4.

3.3.1.3 Concentration-Dependent Inhibition of Diclofenac on Collagen-Stimulated Platelet

Since platelet responses induced by collagen were also sensitive to the effects of diclofenac, the concentration effects of diclofenac against collagen-induced platelet aggregation and adhesion were also determined (Figure 3.9). As for AA, diclofenac inhibited collagen-stimulation of platelets in a concentration-dependent manner, although the effects lessened with higher concentrations of collagen. For instance, 3µg/ml collagen induced platelet aggregation of 70±8%, which decreased following treatment with 0.1, 1, 10, and 100µM diclofenac to 51±9%, 34±7%, 29±10% and 26±8%, respectively. For adhesion induced by 3µg/ml collagen, treatment with the same concentrations of diclofenac reduced platelet adhesion to 26±4%, 5±1%, 17±5% and 20±6% compared to control response of 30±4%.

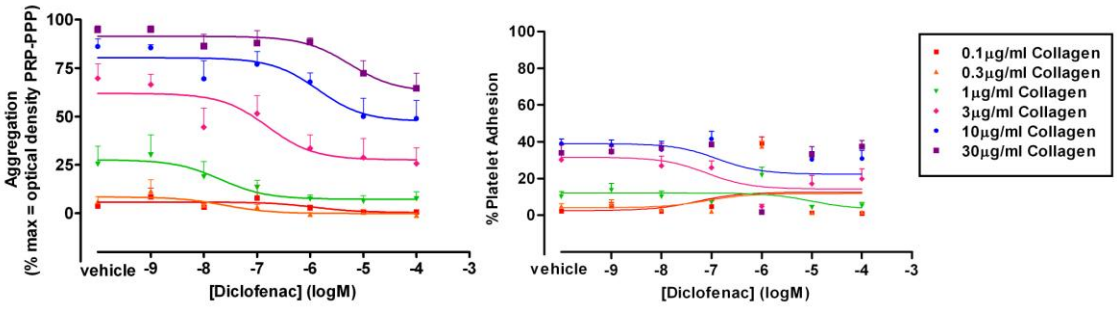


Figure 3.9: *Diclofenac inhibits platelet aggregation stimulated by collagen (COL) in a concentration-dependent manner.* PRP was incubated with various concentrations of diclofenac ranging from 1nM-0.1mM for 30 min at 37^0C before determination of platelet aggregation and adhesion induced by various concentrations of AA. Each data point represents mean ± S.E.M. (n=4).

3.3.1.4 Concentration-Dependent Inhibition of Diclofenac on Combined Collagen and Adrenaline-Stimulated Platelet

The effects of diclofenac were also studied on platelets stimulated by combination of collagen at 1µg/ml and adrenaline at 0.01µM and 0.1µM (Figure 3.10). In platelets stimulated by collagen alone, aggregation was decreased by 0.01 and 0.1 µM diclofenac to 27±4% and 15±3% from 28±5% in control conditions. For the same concentrations of diclofenac, platelet aggregation induced by 0.1µM adrenaline was decreased to 20±7% and 7±2% from 21±7%. Combination of agonists increased platelet aggregation and adhesion and was still sensitive to diclofenac. For example, for collagen combined with 0.01µM adrenaline the aggregation was 33±6% which decreased to 20±4% and 17±3% in the presence of 0.01 and 0.1 µM diclofenac. For collagen combined with 0.1µM adrenaline, the aggregation was reduced by diclofenac to 52±5% and 29±5% from the control level of, 55±7%. Inhibitory effects of diclofenac on platelet adhesion were also seen against both agonist combinations. For instance, collagen and 0.01µM adrenaline induced platelet adhesion of 19±3% which was decreased in the presence of diclofenac 0.01 and 0.1 µM to 12±1% and 9±1%, respectively.

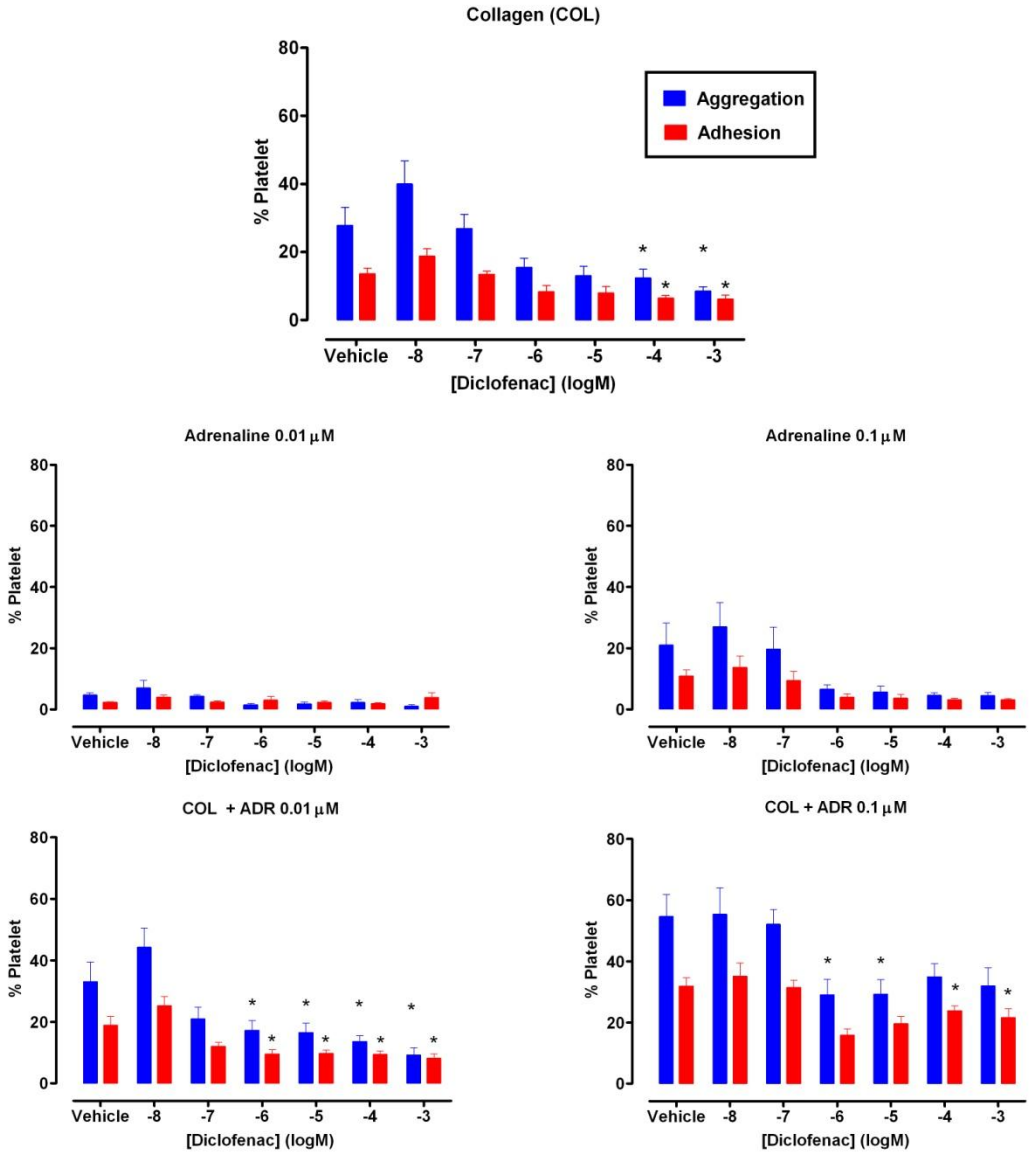


Figure 3.10: *Diclofenac inhibits in a concentration-dependent manner platelet aggregation and adhesion induced by combination of agonists.* PRP was incubated with various concentrations of diclofenac before addition of combined collagen (1µg/ml) and adrenaline (ADR: 0.1 and 0.01µM). Each data point represents mean ± S.E.M., n=4. * indicates P<0.05 as compared to control vehicle determined by one-way ANOVA.

3.3.1.5 Effects of COX-2 Inhibitor, Parecoxib (1mM) on Platelet Activation by Various Agonists

Parecoxib was used to study the effects of selective COX-2 inhibition on platelet activation by various agonists using the methods described above. As expected, there were no changes in platelet aggregation or adhesion noted (Figure 3.11 and Figure 3.12).

3.3.1.6 Effects of COX-2 Inhibitor, Parecoxib, on Platelet Activation by Combination of Collagen and Adrenaline

As above, parecoxib did not affect platelet aggregation or adhesion stimulated by collagen and adrenaline (Figure 3.13).

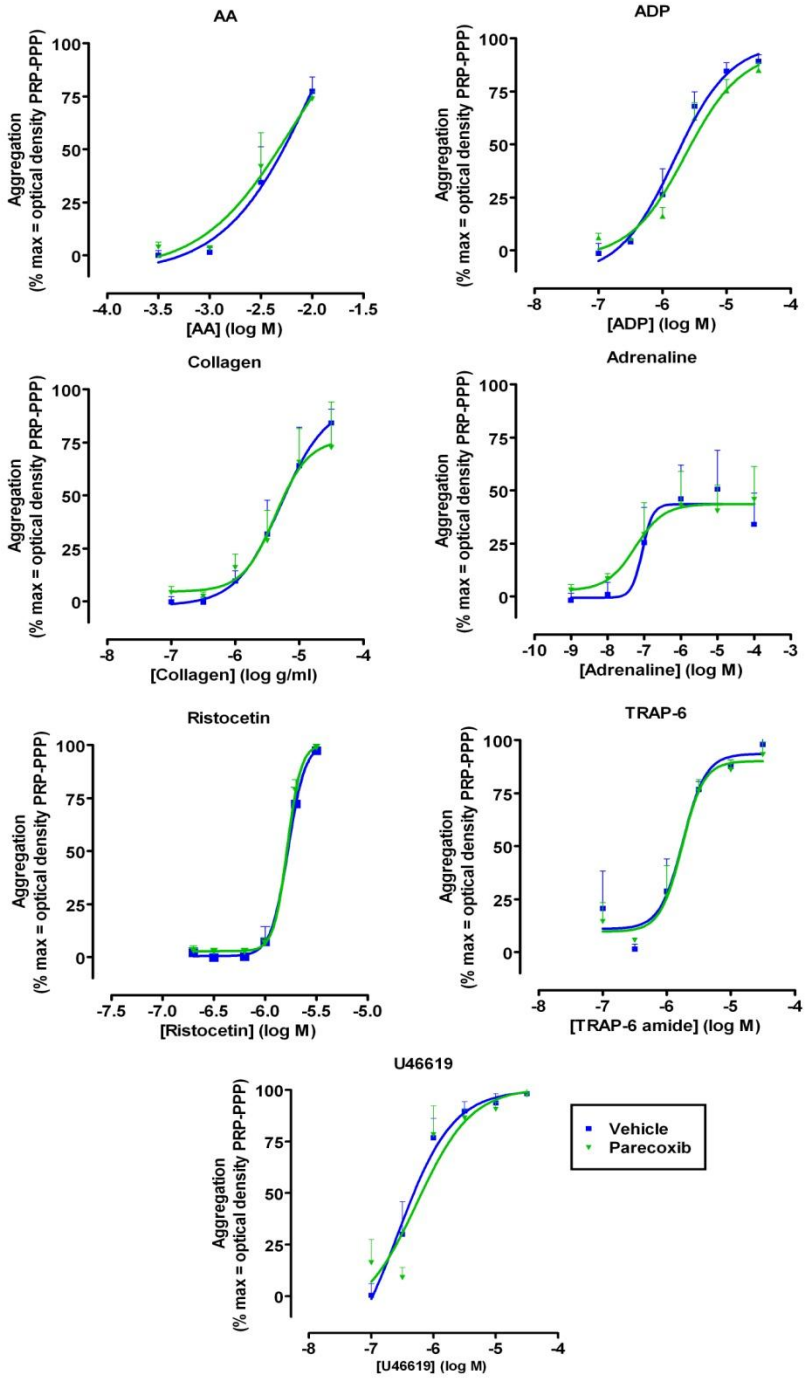


Figure 3.11: *Effects of parecoxib on platelet aggregation stimulated by various agonists.* PRP was incubated with 1mM parecoxib before agonist-stimulated platelet aggregation was determined in 96-well plates. Each data point represents mean ± S.E.M. (n=5).

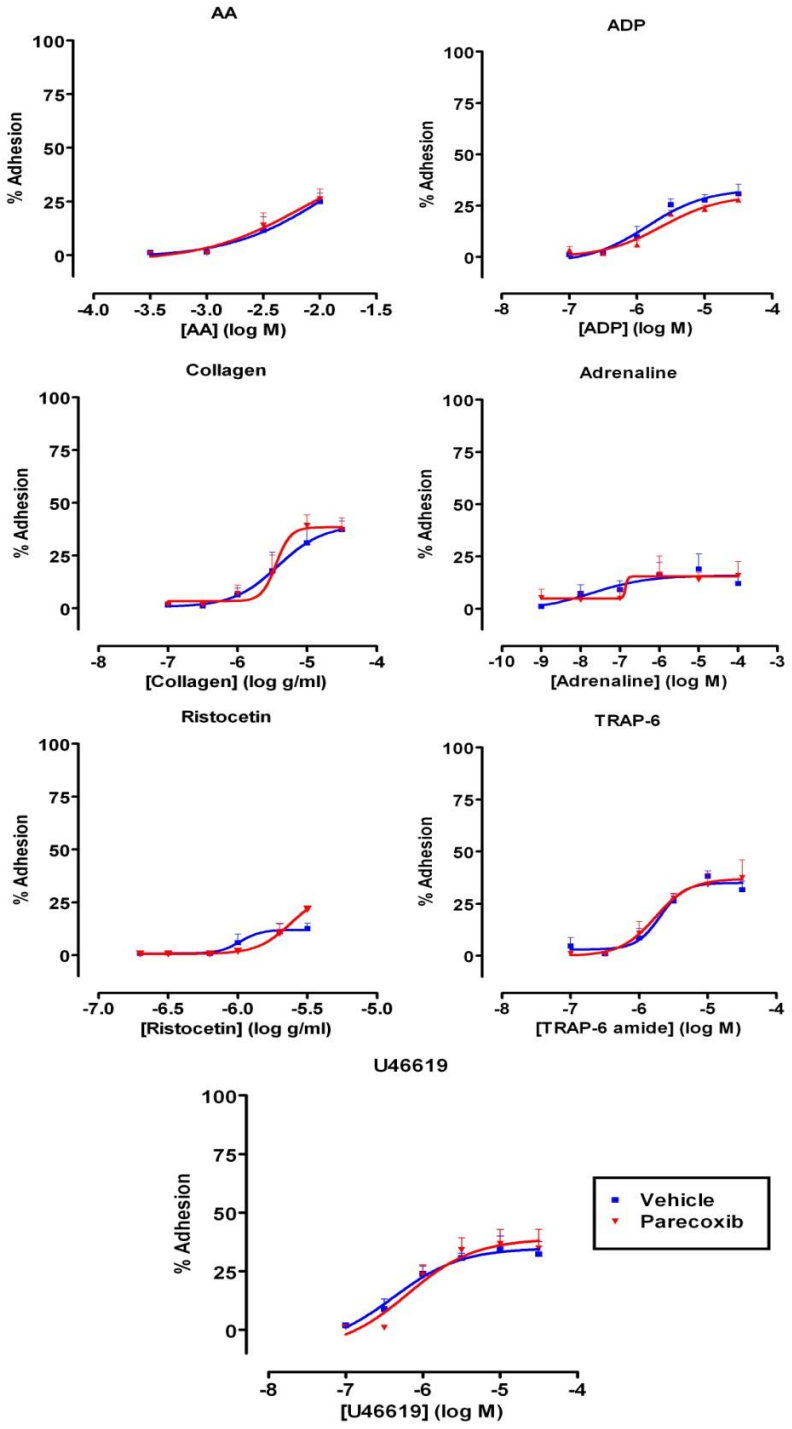


Figure 3.12: *Effects of parecoxib on platelet adhesion stimulated by various agonists.* PRP was incubated with 1mM parecoxib before agonist-stimulated platelet aggregation was determined in 96-well plates. Each data point represents mean ± S.E.M. (n=5).

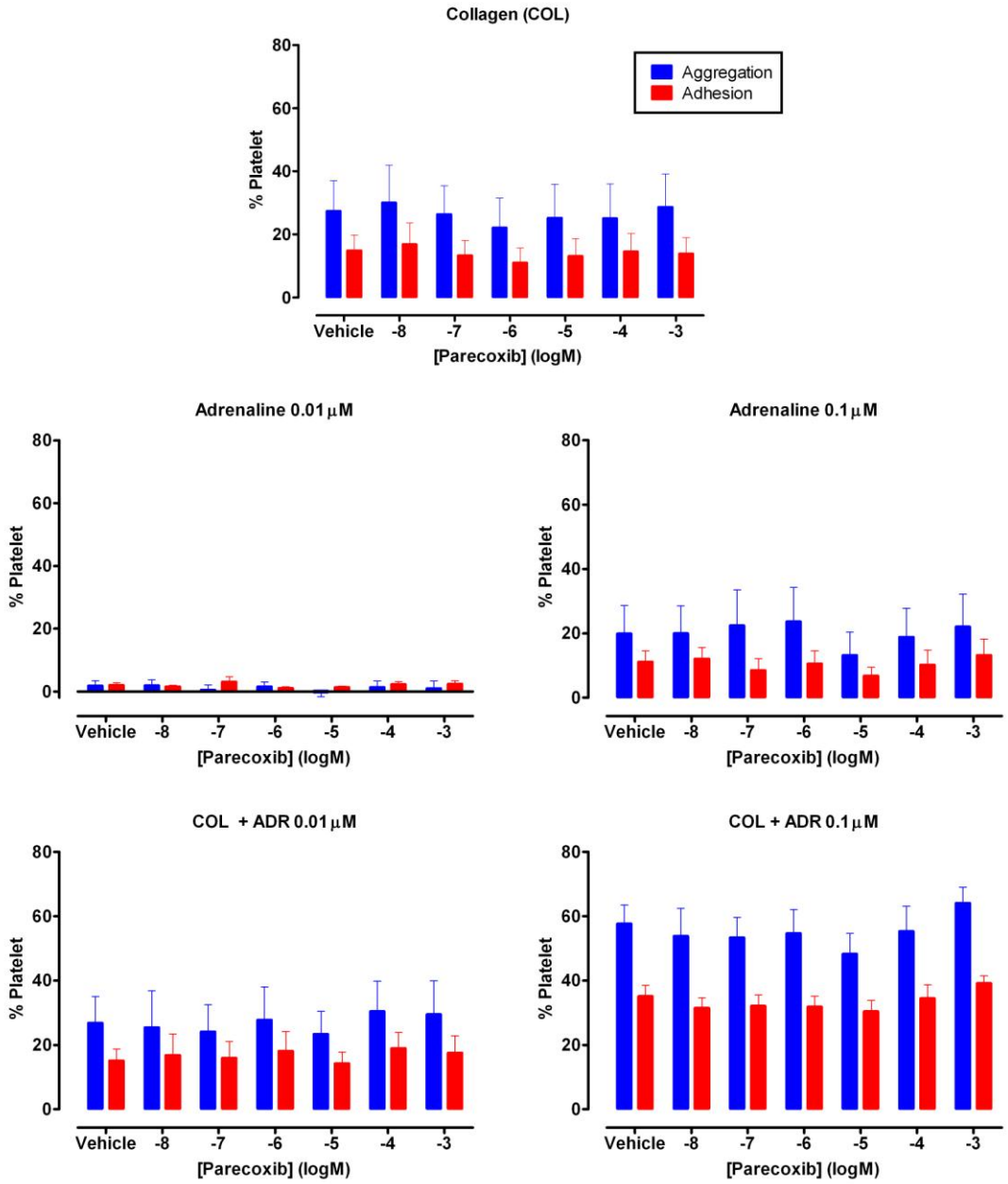


Figure 3.13: *Effects of parecoxib on platelet aggregation and adhesion induced by combined agonists.* PRP was incubated with various concentrations of diclofenac before addition of combination of collagen (1µg/ml) and adrenaline (ADR: 0.1 and 0.01µM). Each data point represents mean ± S.E.M., n=4.

3.3.1.7 Effects of the COX-2 Inhibitor, Lumiracoxib, on Platelet Activation by Various Agonists

Another novel COX-2 selective inhibitor, lumiracoxib was also tested for its effects on platelet function and found to be without effect at concentrations of up to 1mM (Figure 3.14-3.18).

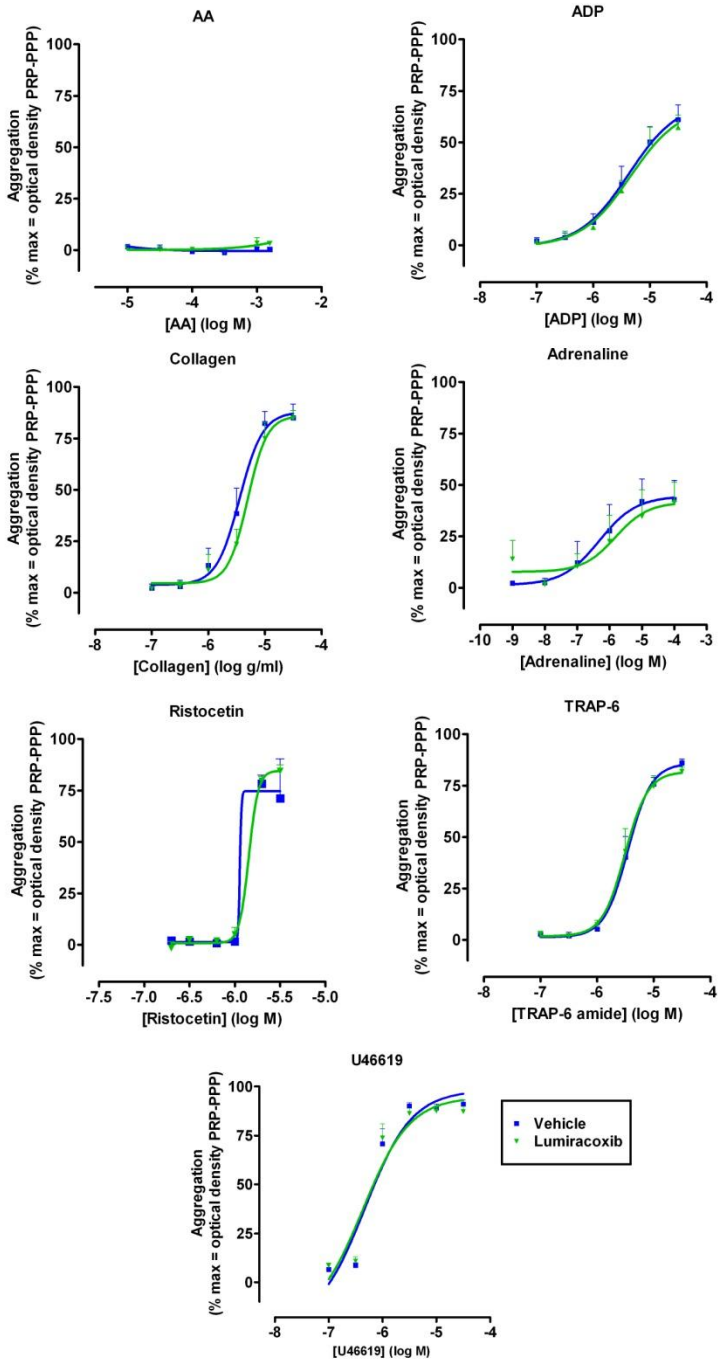


Figure 3.14: *Effects of lumiracoxib on platelet aggregation stimulated by various agonists.* PRP was incubated with 100µM lumiracoxib before agonist-stimulated platelet aggregation was determined in 96-well plates. Each data point represents mean ± S.E.M. (n=4).

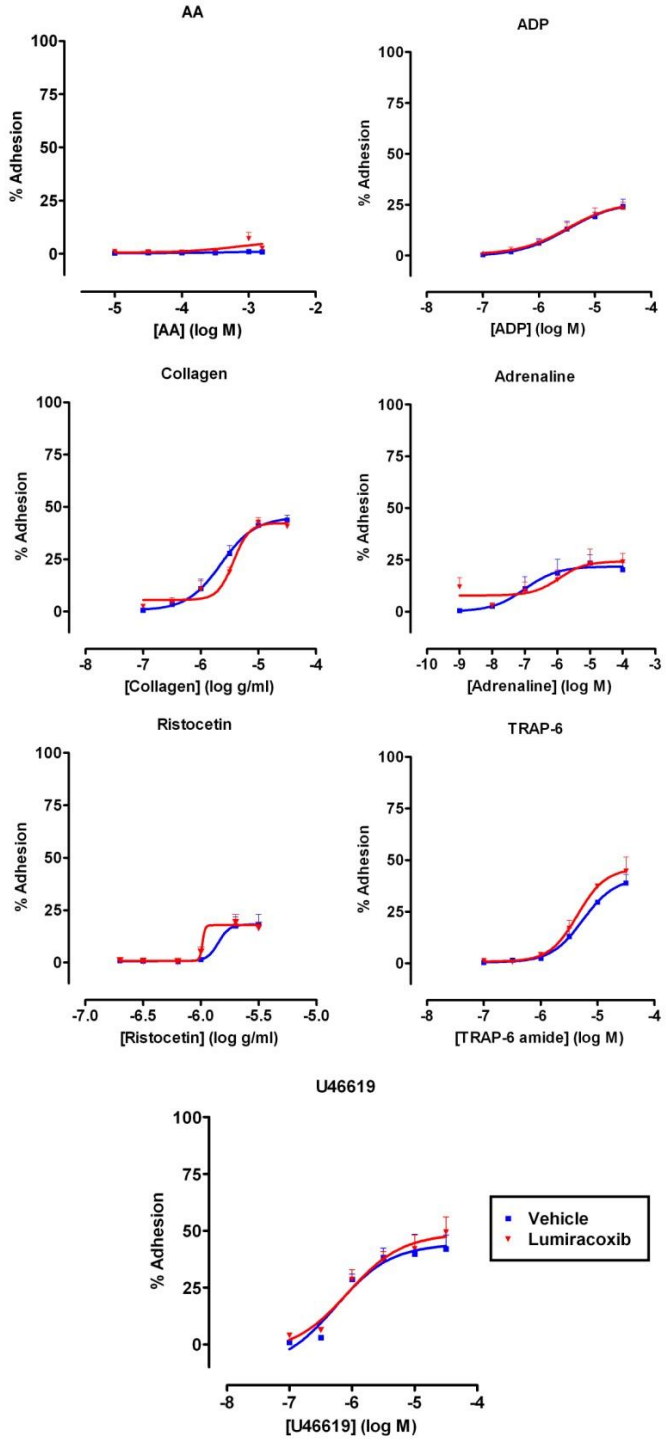


Figure 3.15: *Effects of lumiracoxib on platelet adhesion stimulated by various agonists.* PRP was incubated with 100µM lumiracoxib before agonist-stimulated platelet aggregation was determined in 96-well plate. Each data point represents mean ± S.E.M. (n=4).

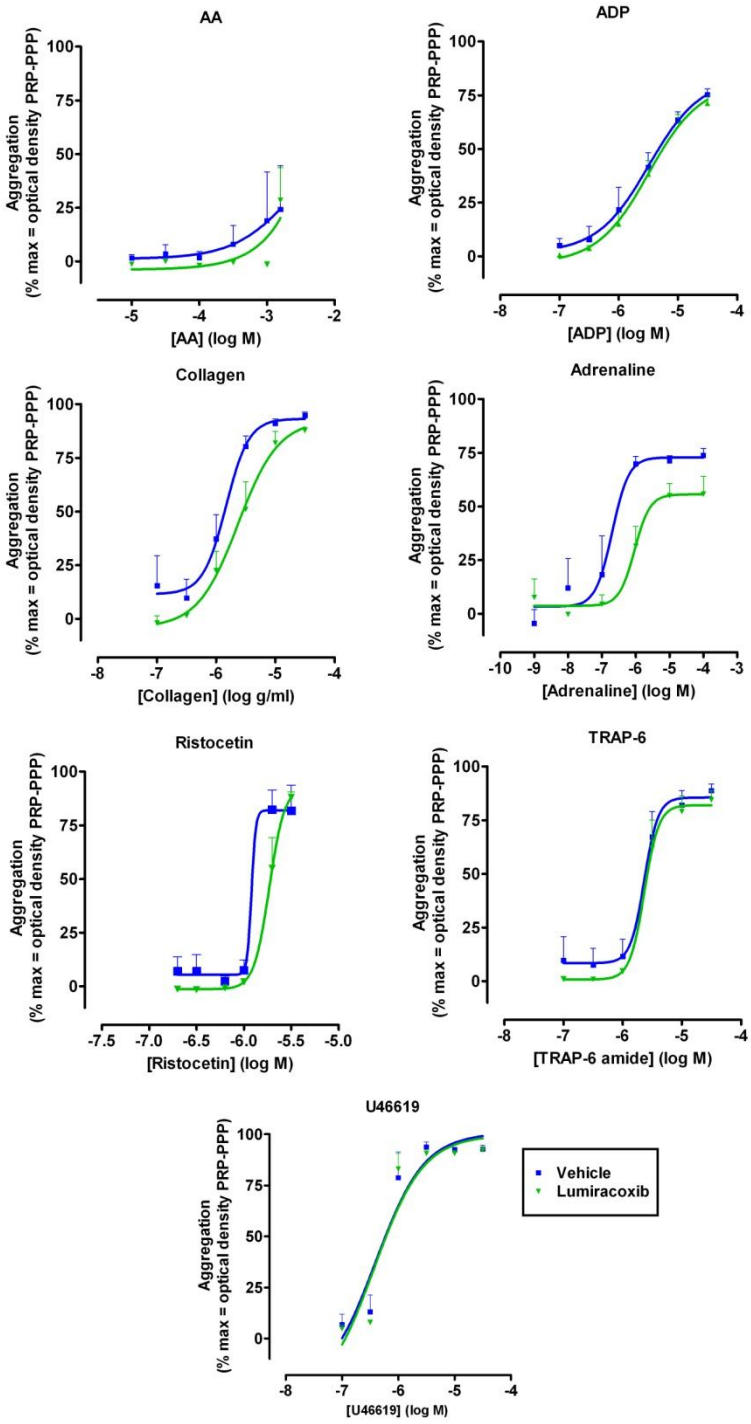


Figure 3.16: *Effects of lumiracoxib on platelet aggregation induced by various agonists.* PRP was incubated with 1mM lumiracoxib before agonist-stimulated platelet aggregation was determined in 96-well plates. Each data point represents mean ± S.E.M. (n=4).

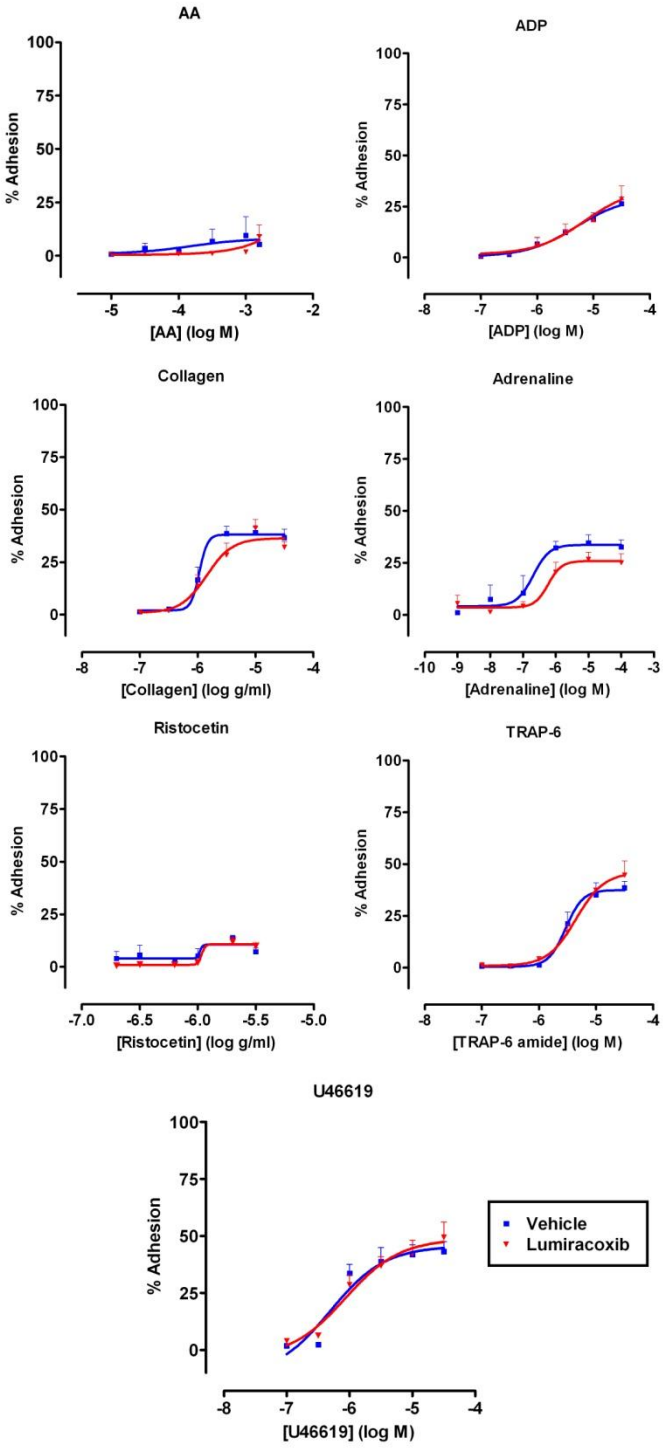


Figure 3.17: *Effects of lumiracoxib on platelet adhesion stimulated by various agonists.* PRP was incubated with 1mM lumiracoxib before agonist-stimulated platelet aggregation was determined in 96-well plates. Each data point represents mean ± S.E.M. (n=4).

3.3.2 Part Two: Effect of Intravenous Diclofenac on Ex Vivo Platelet Function and Other Measurement

3.3.2.1 Effects of intravenous diclofenac on platelet aggregation and adhesion

As shown in Figure 3.18-3.24, diclofenac *in vivo* potently decreased platelet function *ex vivo* as determined by aggregation and adhesion. For example, the aggregation to 1.2mM AA at baseline was 28±9% which was decreased to 9±5% after diclofenac administration (Table 7). Platelet aggregation to 10μM ADP was not affected by administration of normal saline (pre: 56±4%; post: 55±4), however, aggregation was decreased following diclofenac administration to 39±5% from 56±5%. Similar observations were also made for collagen at 10μg/ml, in which platelet aggregation decreased at post-treatment to 28±7% from 68±7% before treatment. Platelet aggregation stimulated by adrenaline and TRAP-6 was also inhibited with diclofenac administration; aggregation stimulated by 100μM adrenaline was inhibited from 51±7% to 18±4% and platelet aggregation stimulated by 30μM TRAP-6 was reduced to 62±5% from 81±3%. There were also significant inhibitions in responses to ristocetin and U46619. Changes in AA-stimulated platelet adhesion caused by diclofenac were not readily observed, probably due to very low baseline percentage of adhesion. However, platelet adhesions stimulated by other agonists were strongly inhibited by diclofenac. For instance, 30μg/ml collagen caused platelet adhesion of 26±4% after diclofenac compared to 43±5% in control conditions (Table 8). Similar observation was found for other agonists, such as ADP, TRAP-6, U46619 and ristocetin.

PLATELET AGGREGATION (%)				
AGONISTS	SALINE		DICLOFENAC	
	PRE	POST	PRE	POST
AA (log M)				
-3.0	16±8	25±10	11±6	7±5
-2.9	15±7	36±9	28±9	9±5
-2.8	26±8	38±8	49±10	32±8
ADP (log M)				
-5.5	37±5	39±4	43±5	31±7
-5.0	56±4	55±4	56±5	39±5
-4.5	70±3	70±4	71±5	56±6
Collagen (log g/ml)				
-5.5	27±6	23±5	44±9	15±7**
-5.0	71±5	66±7	68±7	28±7***
-4.5	87±2	84±4	82±5	40±6***
Adrenaline (log M)				
-6.0	29±6	27±7	30±7	10±4*
-5.0	43±6	38±6	39±6	15±6*
-4.0	53±5	47±6	51±7	18±4***
Ristocetin (log g/ml)				
-6.0	5±1	5±1	25±7	5±4
-5.7	76±4	81±3	71±6	60±7
-5.5	84±5	89±2	83±6	73±6

TRAP-6 (log M)				
-5.5	41±7	39±6	43±7	28±6
-5.0	74±2	73±3	73±2	60±5
-4.5	79±4	81±2	81±3	62±5**
U46619 (log M)				
-5.5	83±3	85±3	75±8	60±9
-5.0	85±1	86±1	83±2	73±7
-4.5	88±1	89±2	86±2	74±7

Table 7: *Inhibitory effects of intravenous diclofenac on platelet aggregation stimulated by various agonists*. Blood samples were taken before and after I.V. administration of diclofenac sodium or saline. Data shown is for top three concentrations of each agonist tested. Each data represents mean ± S.E.M. from 8 different healthy volunteers. * = P<0.05; ** = P<0.01 and *** = P<0.001 determined by one-way ANOVA.

PLATELET ADHESION (%)				
AGONISTS	SALINE		DICLOFENAC	
	PRE	POST	PRE	POST
AA (log M)				
-3.0	6±3	2±1	3±2	14±6
-2.9	4±2	6±1	7±3	7±3
-2.8	6±2	7±3	6±3	7±3
ADP (log M)				
-5.5	17±3	13±3	18±3	11±3
-5.0	29±3	22±3	26±3	20±4
-4.5	37±3	34±3	36±4	26±4
Collagen (log g/ml)				
-5.5	18±4	13±3	21±4	9±4
-5.0	46±4	41±4	40±5	20±5
-4.5	45±2	44±3	43±5	26±4
Adrenaline (log M)				
-6.0	15±3	11±4	13±2	8±4
-5.0	25±4	18±4	22±3	12±4
-4.0	32±3	25±4	28±4	14±4
Ristocetin (log g/ml)				
-6.0	3±1	2±0	8±2	2±2
-5.7	35±3	35±5	37±5	25±5
-5.5	35±4	29±3	44±5	23±4

TRAP-6 (log M)				
-5.5	16±3	16±4	19±3	12±4
-5.0	43±3	38±3	44±3	35±4
-4.5	50±3	49±4	50±5	36±4
U46619 (log M)				
-5.5	57±3	54±2	56±5	40±7
-5.0	64±4	63±4	63±6	52±6
-4.5	66±6	65±5	62±6	52±6

Table 8: *Inhibitory effects of intravenous diclofenac on platelet adhesion stimulated by various agonists*. Blood samples were taken before and after I.V. administration of diclofenac sodium or saline. Data shown is for top three concentrations of all agonists tested. Each data represents mean ± S.E.M. from 8 different healthy volunteers. Data has been analysed by one-way ANOVA.

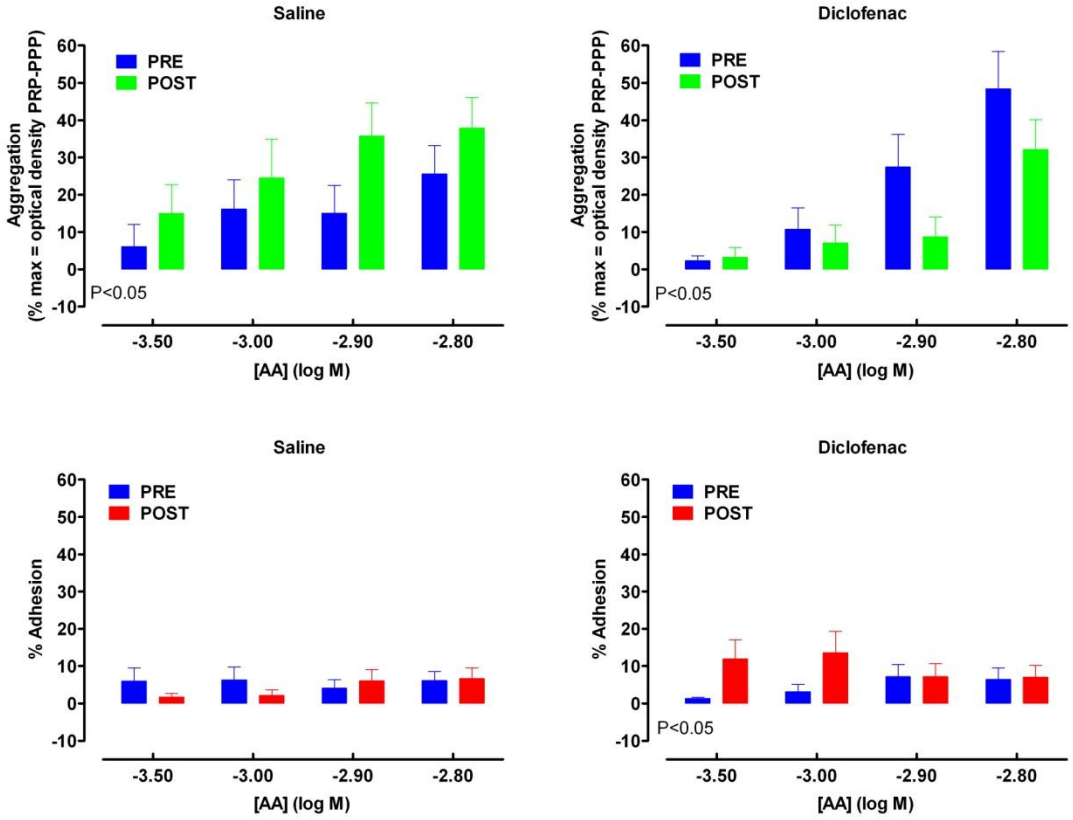


Figure 3.18: *Inhibitory effects of intravenous diclofenac on AA-stimulated platelet aggregation and adhesion*. Blood samples were taken before and after I.V. administration of diclofenac sodium or saline. Each data represents mean ± S.E.M. of data from 8 different healthy volunteers. Two-way ANOVA was performed to compare between pre and post-treatment.

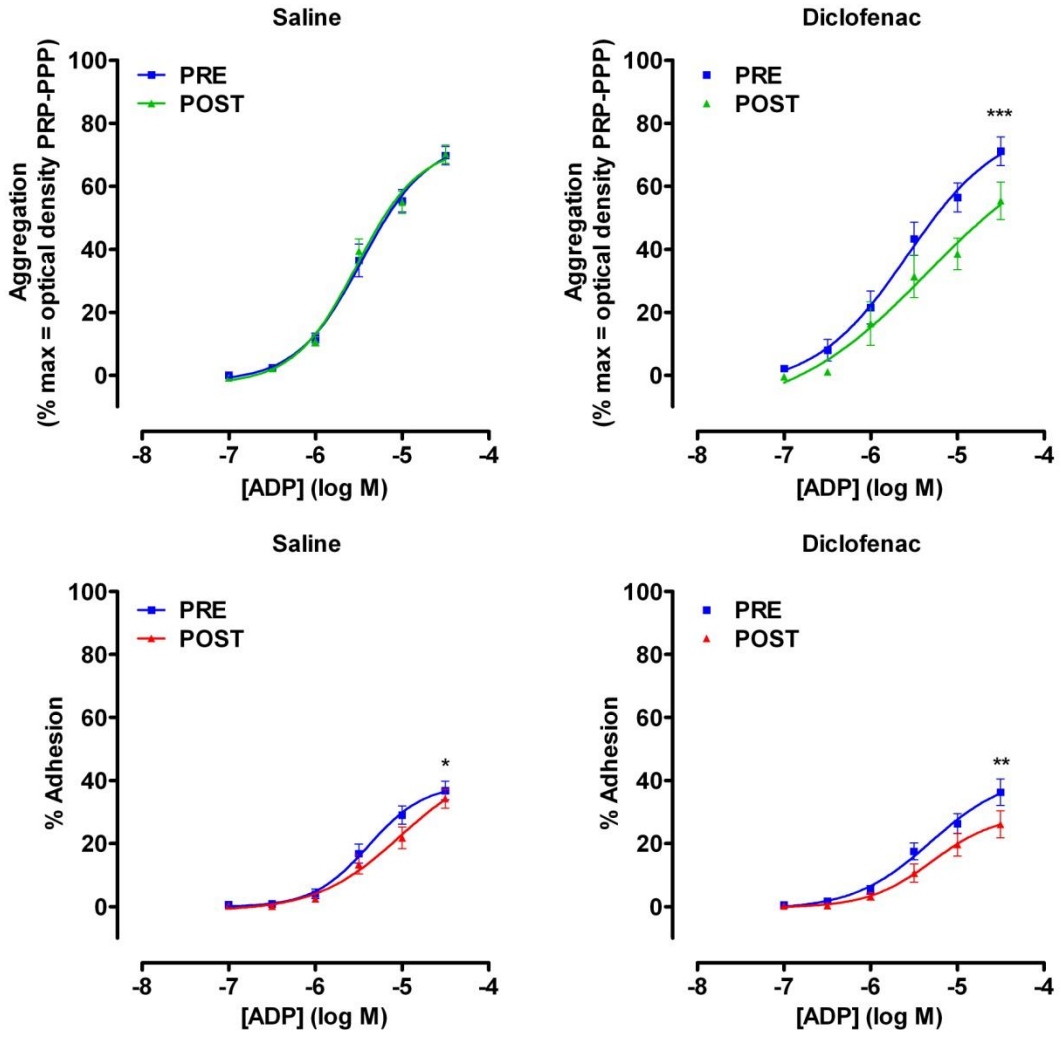


Figure 3.19: *Inhibitory effects of intravenous diclofenac on ADP-stimulated platelet aggregation and adhesion*. Blood samples were taken before and after I.V. administration of diclofenac sodium or saline. Each data point represents mean ± S.E.M. from 8 different healthy volunteers. Two-way ANOVA was used to compare between pre and post-treatment in which * = P<0.05, ** = P<0.01 and *** = P<0.001.

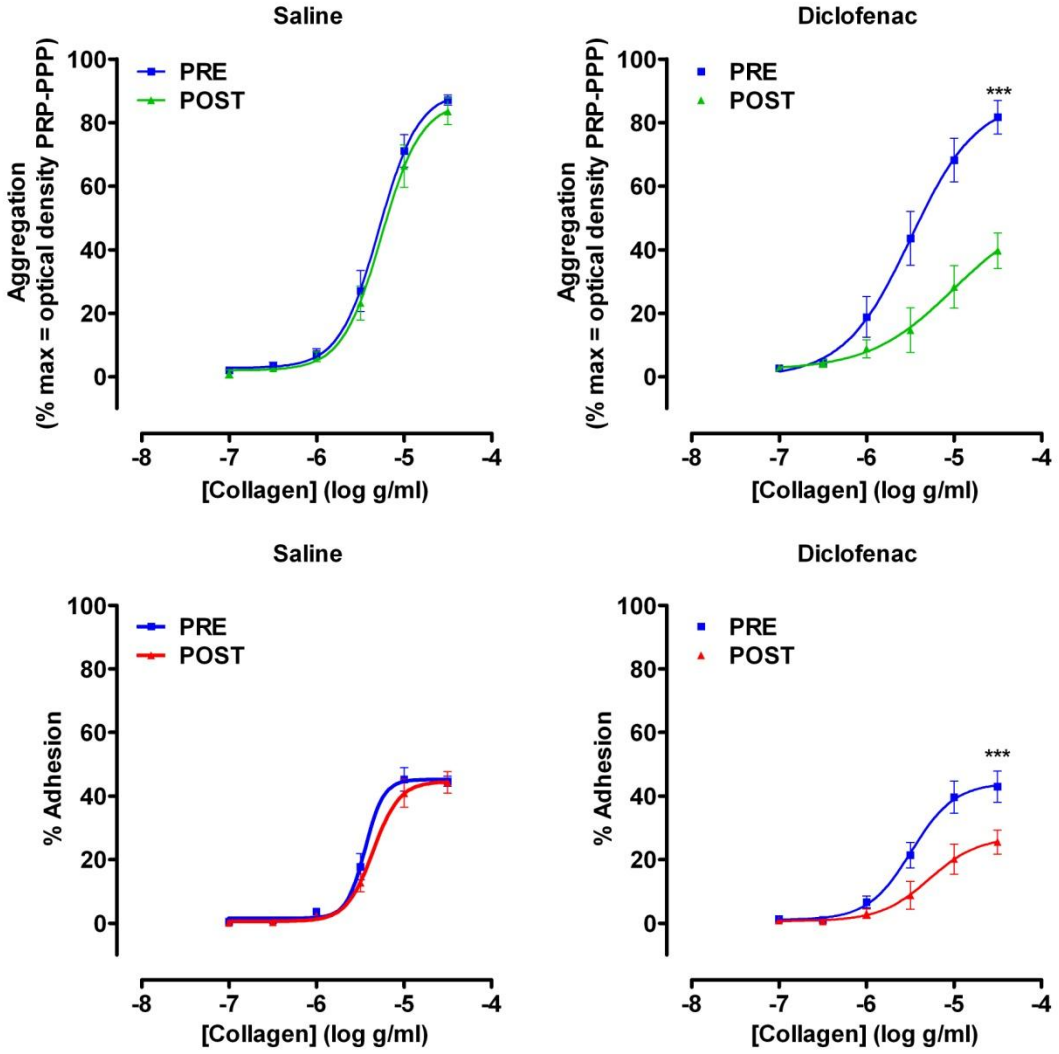


Figure 3.20: *Inhibitory effects of intravenous diclofenac on collagen-stimulated platelet aggregation and adhesion*. Blood samples were taken before and after I.V. administration of diclofenac sodium or saline. Each data point represents mean ± S.E.M. from 8 different healthy volunteers. Two-way ANOVA was used to compare between pre and post-treatment in which * = P<0.05, ** = P<0.01 and *** = P<0.001.

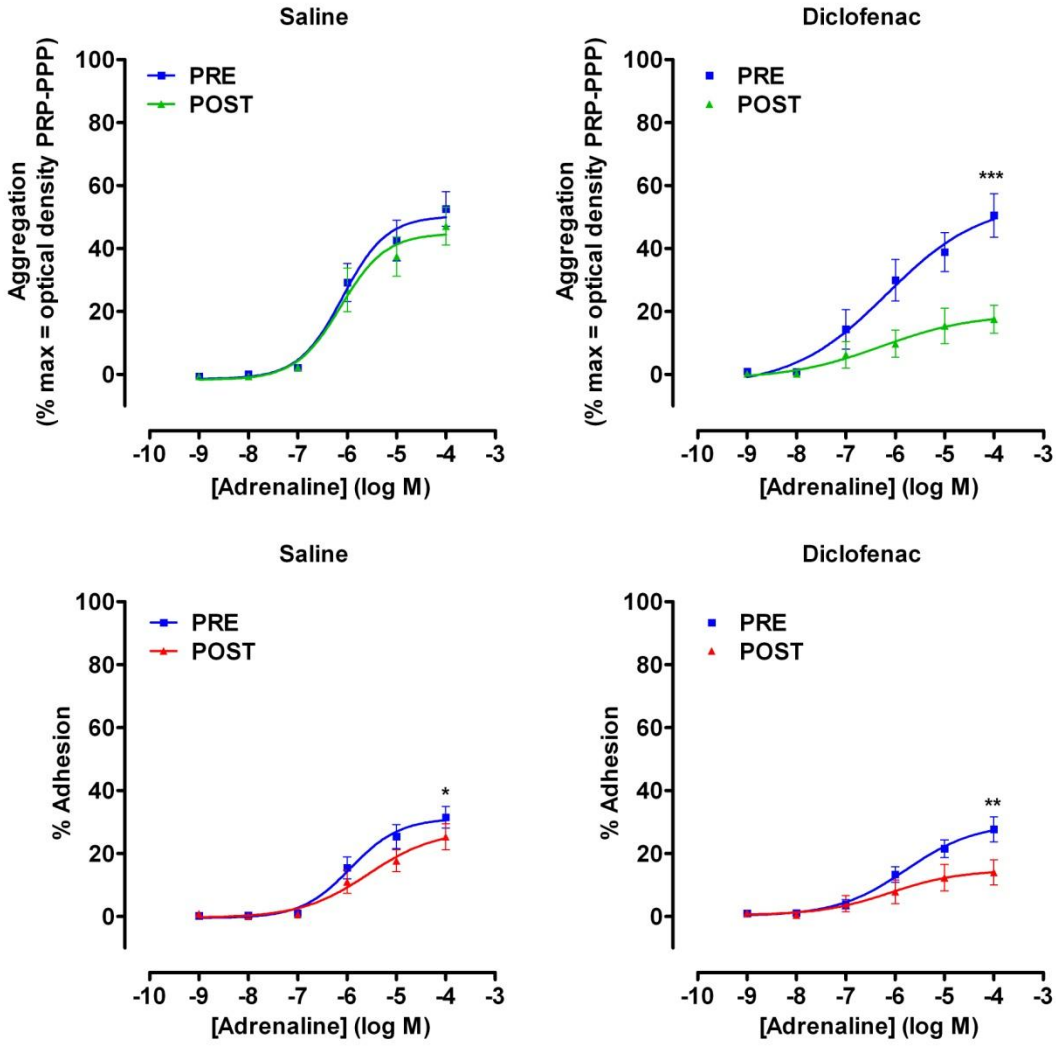


Figure 3.21: *Inhibitory effects of intravenous diclofenac on adrenaline-stimulated platelet aggregation and adhesion*. Blood samples were taken before and after I.V. administration of diclofenac sodium or saline. Each data point represents mean ± S.E.M. from 8 different healthy volunteers. Two-way ANOVA was used to compare between pre and post-treatment in which * = P<0.05, ** = P<0.01 and *** = P<0.001.

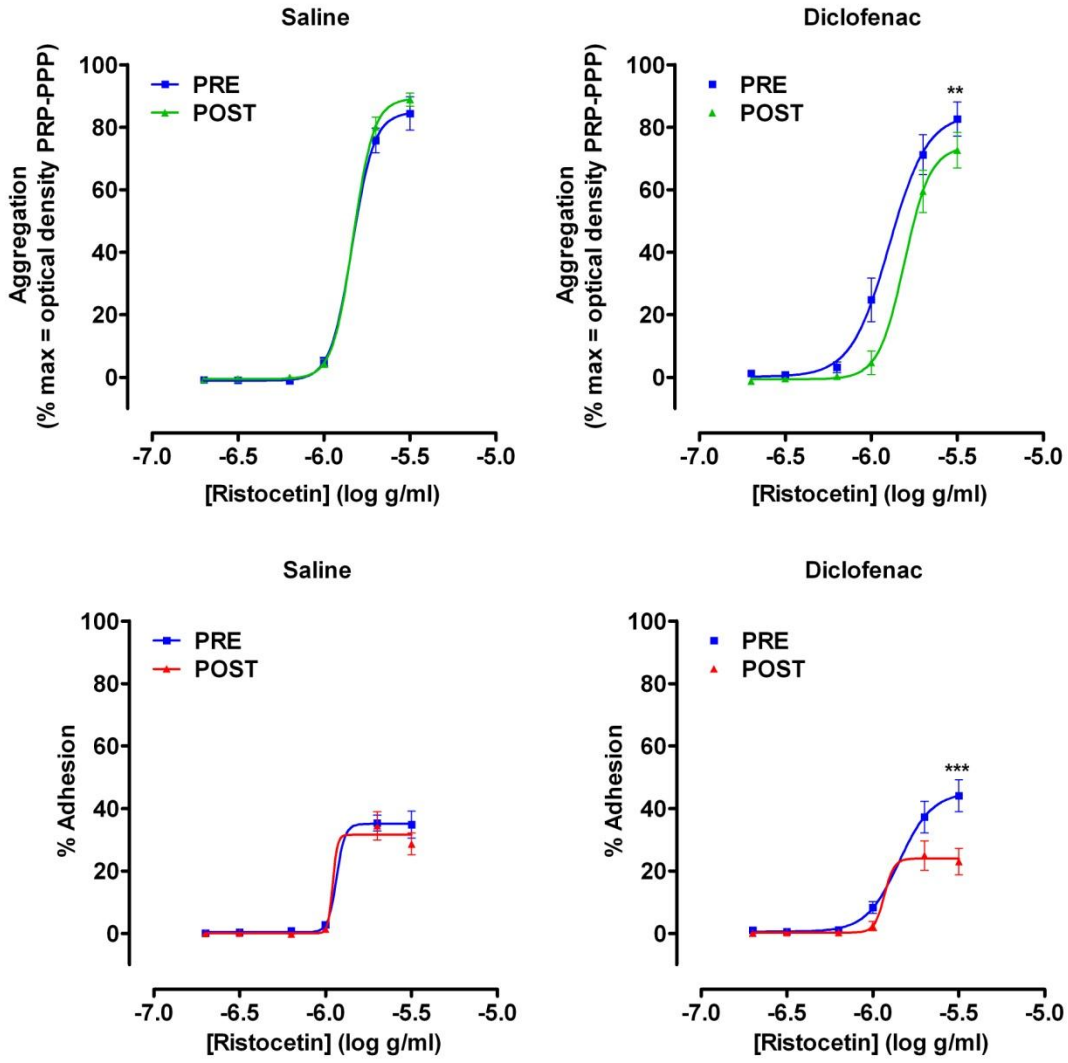


Figure 3.22: *Inhibitory effects of intravenous diclofenac on ristocetin-stimulated platelet aggregation and adhesion*. Blood samples were taken before and after I.V. administration of diclofenac sodium or saline. Each data point represents mean ± S.E.M. from 8 different healthy volunteers. Two-way ANOVA was used to compare between pre and post-treatment in which * = P<0.05, ** = P<0.01 and *** = P<0.001.

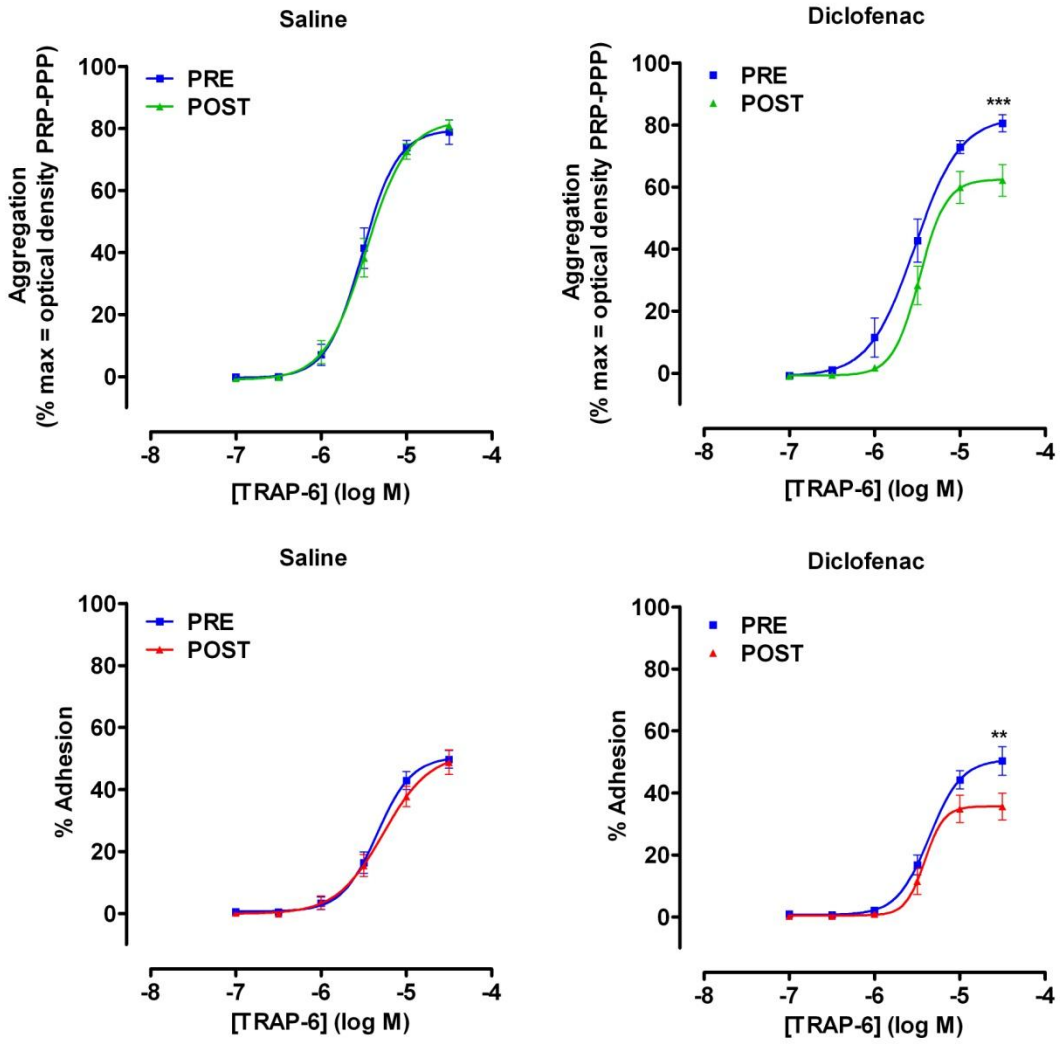


Figure 3.23: *Inhibitory effects of intravenous diclofenac on TRAP-6 -stimulated platelet aggregation and adhesion*. Blood samples were taken before and after I.V. administration of diclofenac sodium or saline. Each data point represents mean ± S.E.M. from 8 different healthy volunteers. Two-way ANOVA was performed to compare between pre and post-treatment in which * = P<0.05, ** = P<0.01 and *** = P<0.001.

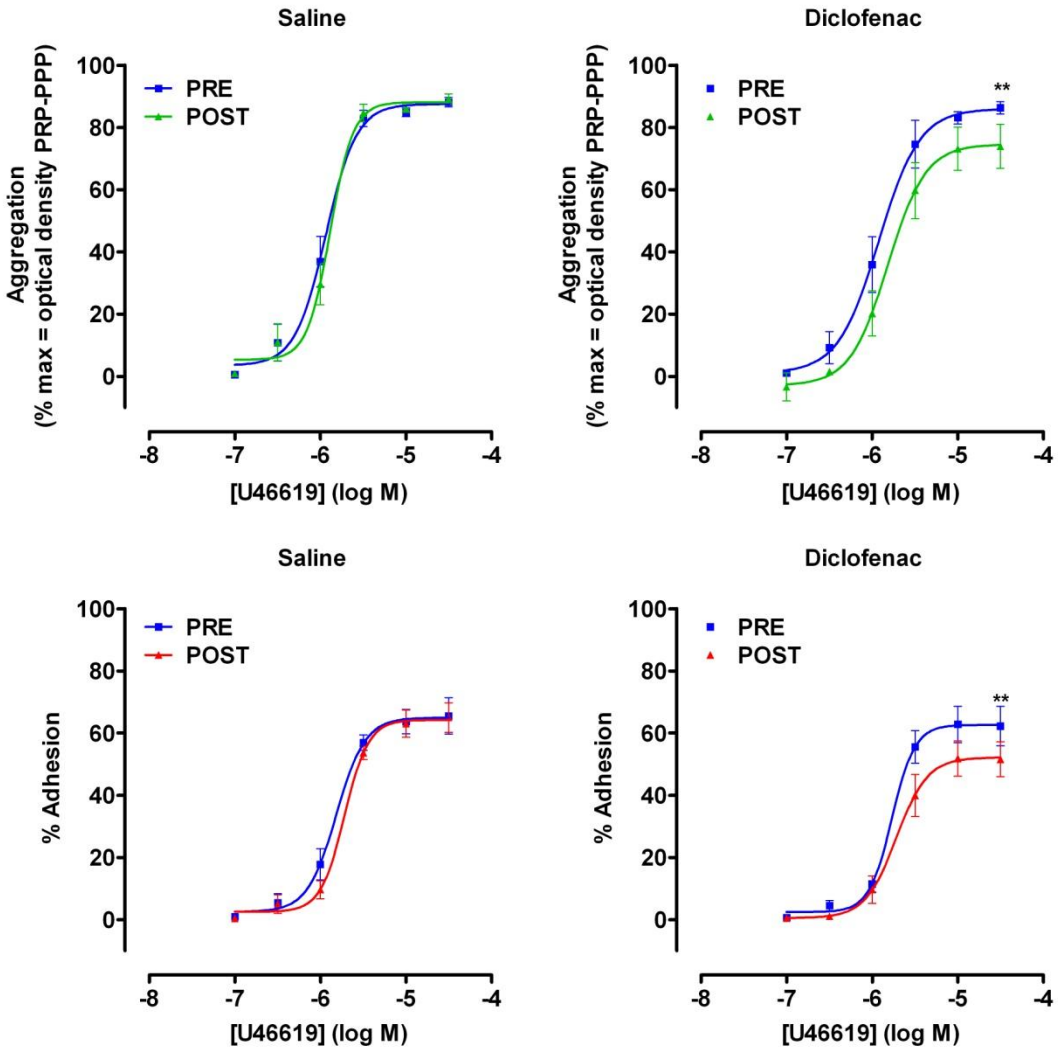


Figure 3.24: *Inhibitory effects of intravenous diclofenac on U46619-stimulated platelet aggregation and adhesion*. Blood samples were taken before and after I.V. administration of diclofenac sodium or saline. Each data point represents mean ± S.E.M. from 8 different healthy volunteers. Two-way ANOVA was performed to compare between pre and post-treatment in which * = P<0.05, ** = P<0.01 and *** = P<0.001.

3.3.2.2 Determination of Blood TXB_2 and PGE_2 Level Following Administration of Diclofenac

Radioimmunoassays were performed to investigate the changes in the levels of TXB_2 and PGE_2 in the blood following I.V. administration of diclofenac sodium. Blood were taken at various time points before and after hyperaemia to study the changes of eicosanoids. As shown in Figure 3.25, there were no significant changes of TXB_2 production in non-stimulated blood when compared between baseline, pre- and post hyperaemia in control saline and diclofenac treatment. When stimulated by calcium ionophore, baseline levels of TXB_2 were increased as shown in Figure 3.26, which were clearly decreased at post-hyperemia in both saline and diclofenac administration. PGE_2 levels were clearly and significantly suppressed following intravenous diclofenac administration suggesting inhibition of blood COX-2 activity (Figure 3.27).

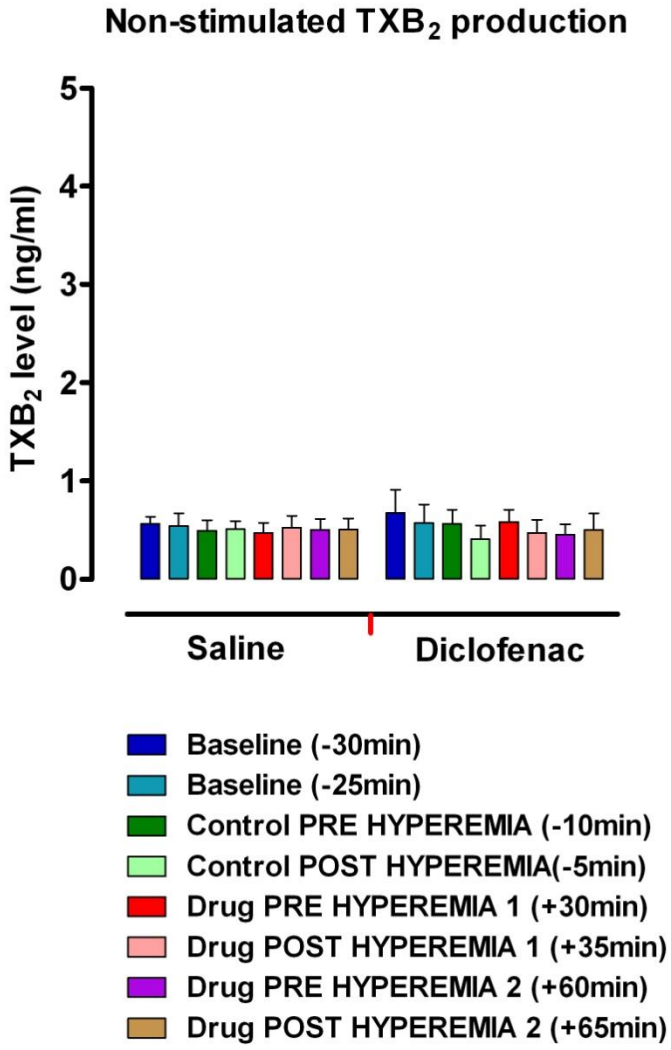


Figure 3.25: *Effects of intravenous diclofenac on TXB_2 production in non-stimulated blood.* TXB_2 levels were measured by radioimmunoassay before and after diclofenac sodium or saline administration. Each data point represents mean ± S.E.M. from 8 different healthy volunteers.

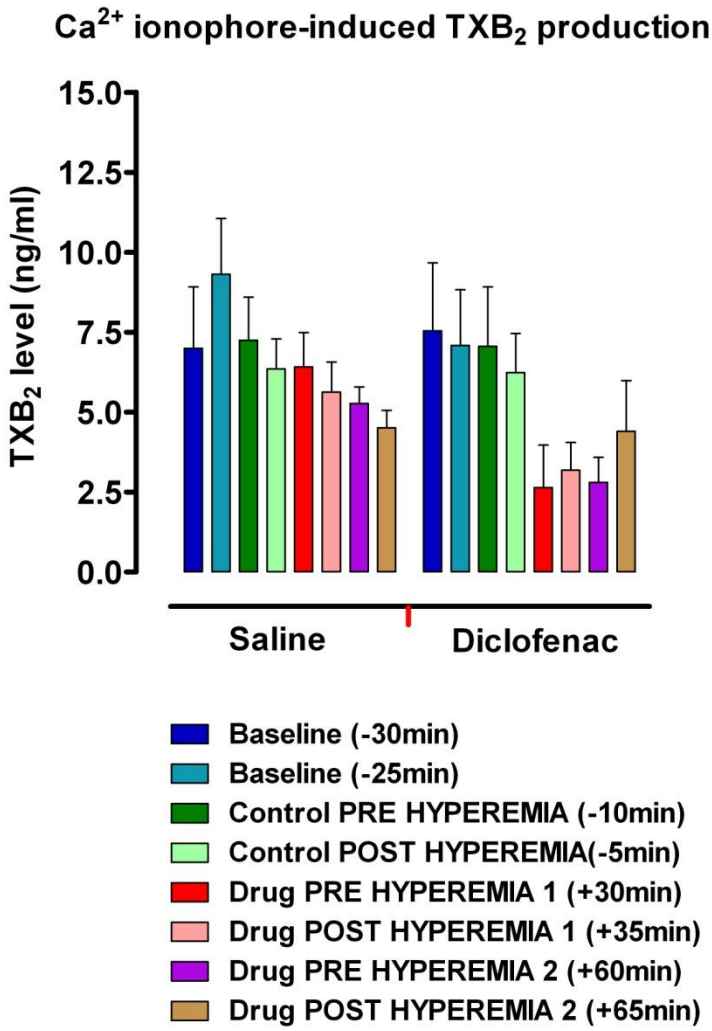


Figure 3.26: *Effects of intravenous diclofenac on TXB_2 production in calcium ionophore-stimulated blood.* TXB_2 levels were measured by radioimmunoassay before and after diclofenac sodium or saline administration. Each data point represents mean ± S.E.M. from 8 different healthy volunteers.

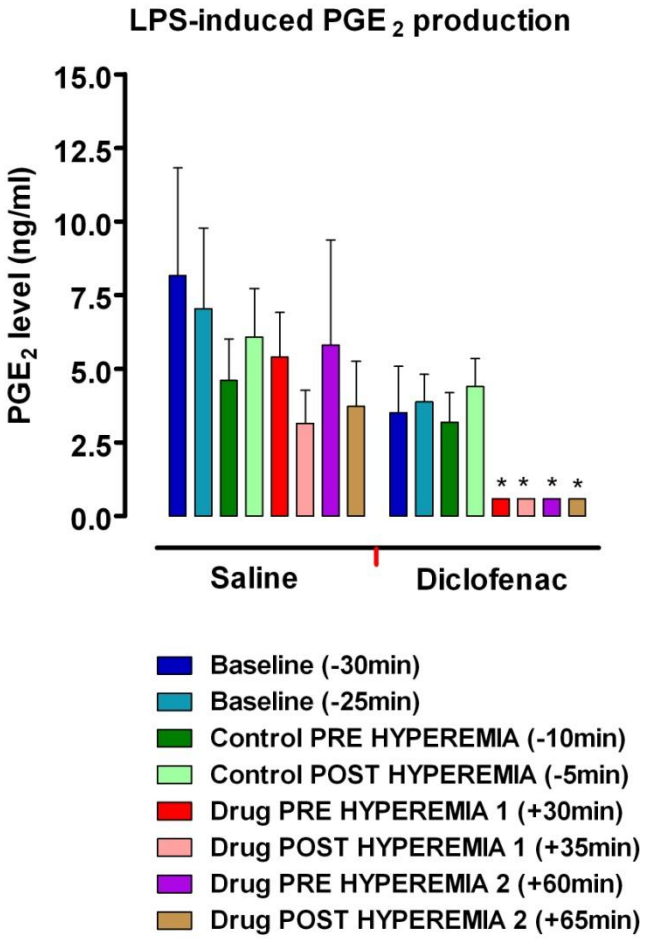


Figure 3.27: *Effects of intravenous diclofenac on PGE_2 production in LPS-stimulated blood.* PGE_2 levels were measured by radioimmunoassay before and after diclofenac sodium or saline administration at different time point. Each data point represents mean ± S.E.M. from 8 different healthy volunteers. * indicates $P<0.05$ by one-way ANOVA.

3.3.2.3 Effects of Intravenous Diclofenac on PFA-100 CADP and CEPI Cartridge Closure Time (CT) Measured by PFA-100

Further study of platelet function was made before and after administration of diclofenac or control saline as measured by Platelet Function Analyzer 100 (PFA 100) as shown in Figure 3.28. No significant changes of CADP closure times followed administration of control saline or diclofenac. For CEPI closure times, no changes followed administration of control saline; however, CEPI closure times were increased by administration of diclofenac to 181±18 seconds from 149±14 seconds.

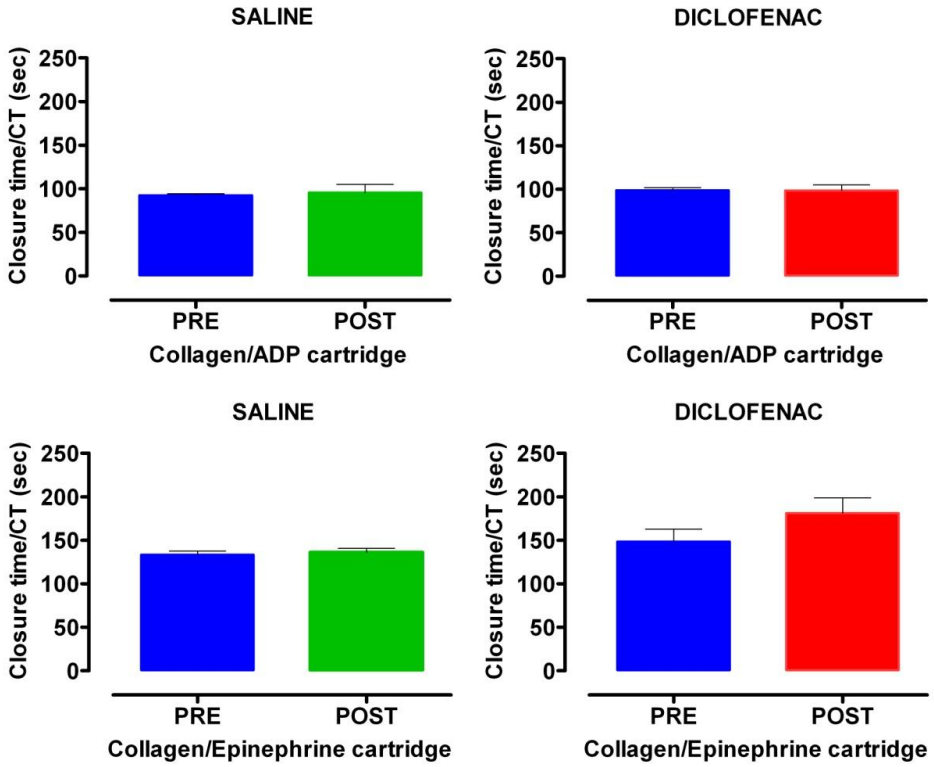


Figure 3.28: *Effects of intravenous diclofenac on platelet function measured by PFA-100.* Closure times (sec) for both collagen/ADP and collagen/epinephrine cartridges were measured before and after administration of diclofenac sodium or saline. Each data represent mean ± S.E.M. from 6 individual healthy volunteers. Statistical analysis student t-test was performed to compare between pre- and post-treatment.

3.4 Discussion

This study confirms previous clinical trials that have shown inhibition of platelet function by NSAIDs. A study in healthy male volunteers by Bauer *et al.* (2010) found that diclofenac impaired platelet function as measured by platelet count and PFA-100, but to a lesser extent than preferential COX-1 inhibitors such as aspirin and ketorolac. The current study also shows that parecoxib did not exert an inhibitory effect on platelet *in vitro*. Parecoxib (Dynastat) is an amide prodrug of valdexocib, an oral COX-2 inhibitor and is the first injectable COX-2 inhibitor for the management of pain (Graff *et al.*, 2007). Parecoxib is converted *in vivo* to valdecoxib and propionic acid, with its $t_{1/2}$ being approximately 30 minutes. Earlier work has found that parecoxib produces longer analgesic effects than diclofenac in patients undergoing elective general surgery. However, platelet aggregation measured by ADP-induced optical aggregometry was more strongly inhibited by diclofenac than parecoxib (Bajaj *et al.*, 2004). This study suggested an advantage of parecoxib over diclofenac in that it could provide more effective analgesic and anti-inflammatory effects without increasing the risk of bleeding complications following inhibition of platelet COX-1. A previous study has also shown that parecoxib did not affect platelet function or TxB_2 formation in patients undergoing routine partial menisectomy (Graff *et al.*, 2007). Similarly, another COX-2 inhibitor, rofecoxib did not effects PFA-100 closure time whereas aspirin, diclofenac and lornoxicam significantly prolong the CTs in healthy volunteers receiving the drugs orally (Blaicher *et al.*, 2004). Conversely, another study has shown

that parecoxib enhanced shear stress-induced platelet aggregation in the presence of an arterial stenosis in rats (Borgdorff *et al.*, 2006).

In addition, lumiracoxib (Prexige®), a novel COX-2 selective inhibitor use in the treatment of osteoarthritis, rheumatoid arthritis and acute pain was also studied for its platelet inhibitory effects *in vitro*. With regard to its chemical structure, lumiracoxib is different from other COX-2 inhibitors, which are typically sulphonamides (celecoxib and valdecoxib) or sulfones (rofecoxib and etoricoxib). In this study, lumiracoxib exhibited no effect on platelet function *in vitro* consistent with a selective effect upon COX-2 inhibitor with no influence on platelet COX-1 and the formation of TXA_2.

It is vital to understand the role of COX-1 and COX-2 products in maintaining normal physiology before the side effects of both NSAIDs and COX-2 inhibitors are explored. COX-1 exists in most tissues and is responsible for maintaining normal gastric mucosa and regulates platelet function (Konstam *et al.*, 2002). In contrast, COX-2 is nearly undetectable in non-stimulated cells but is inducible in the presence of pro-inflammatory cytokines and produces prostaglandins that are involved in pain and inflammatory reaction (Urban, 2000). Traditional NSAIDs inhibits both COX-1 and COX-2, thus decreasing the biosynthesis of prostaglandins and other proinflammatory agents explaining their therapeutic and toxic effects (Vane, 1971). It appears in general terms that COX-1 inhibition in gastric mucosa and decreases of PGI_2 and PGE_2 synthesis leads to the loss of a local protective effect of GIT (Emery, 1996). This hypothesis supported the development

of selective COX-2 inhibitors that should have less GIT toxicity and produce less bleeding than traditional NSAIDs.

Another concern is that COX-2 inhibitors may cause an increase in the risk of cardiovascular disease due to their inhibition of vascular PGI_2 synthesis (Connolly, 2003). Although The Vioxx Gastrointestinal Outcomes Research (VIGOR) trial was initially carried out to investigate the GIT adverse events caused by rofecoxib or naproxen, cardiovascular findings in this research suggested the potential for prothrombotic effects of rofecoxib (Bombardier *et al.*, 2000). As the incidence of myocardial infarction was found similar in patients taking rofecoxib in the VIGOR study and celecoxib in the Celecoxib Long-term Arthritis Safety Study (CLASS), Mukherjee *et al.* (2001) suggested an association between the use of coxibs and an increased risk of cardiovascular events such as myocardial infarction (Mukherjee *et al.*, 2001). It is widely understood that COX-2 selective inhibition has no effect on functional platelet COX-1, but it may have reduce the production of vascular prostacyclin, thereby produce an imbalance between TXA_2 and PGI_2, thus promoting a prothrombotic state (McAdam *et al.*, 2005). However, more evidence is required to explore the effects of COX-2 inhibitors aligned with their different pharmacokinetic and pharmacodynamic profiles in regard to the risk of cardiovascular adverse effects. Nevertheless, it was suggested that based on the previous studies and analyses, COX-2 selective inhibitors at a standard recommended doses do not increase the risk of thrombotic events more than do traditional NSAIDs (Warner *et al.*, 2004).

There are many point-of-care type instruments such as VerifyNow and Platelet Function Analyser (PFA-100, Dade-Behring) that can be used to measure platelet function, as well as other methods such as optical aggregometry. PFA-100 is very useful in determining the function of blood platelets under similar conditions to those in the circulation, as this method uses whole blood in a high-shear stress system (Watala *et al.*, 2003). Although PFA-100 has been classified as a method that shows high sensitivity to a variety of clinical situations, such as vWF dysfunction and von Willebrand disease, there is no definite conclusion as to which PFA-100 closure time reflects the ability of platelets to aggregate and adhere (Watala *et al.*, 2003). As prolongation of CTs reflects the degree of platelet inhibition, our data shows that there is no change in the PFA-100 occlusion time of collagen-ADP (CADP) cartridges after administration of diclofenac to the volunteers. However, inhibition of aggregation and adhesion of ADP-induced PRP were detected when using our 96-well plate format method. Further, our data shows that collagen and adrenaline-induced aggregation and adhesion were in agreement with CEPI CTs which shows an inhibition following diclofenac treatment. Previous study also showed that PFA-100 CEPI CT and adrenaline-induced aggregometry is the pair of methods with the higher agreement in monitoring of platelet dysfunction due to aspirin treatment (Tsantes *et al.*, 2008). However, the same study also reported that the PFA-100 CADP CT were not consistent with ADP-induced aggregometry which suggested there are some variables affected both methods in different way.

It is noteworthy that Tsantes *et al.* (2008) have used AA, adrenaline and ADP as agonists in optical aggregometry to compare with PFA-100 CTs whilst Malinin *et al.* (2003) used only adrenaline-induced conventional aggregometry. In parallel, both studies have found that despite the use of different agonists in aggregometry, both aggregometry and PFA-100 seems to be reliable in reflecting the various responses of anti-aggregating agents. The correlation between platelet counts and the PFA-100 measurements is not well-known, however, CADP CT in patients with thrombocytosis not taking aspirin was statistically significantly inversely correlated to platelet count (Tsantes *et al.*, 2008). Meanwhile, our 96-well plate format method is not affected by platelet count in measuring platelet function in response to various agonists (Armstrong *et al.*, 2008). In addition, several studies have suggested that the PFA-100 is more reliable and sensitive than optical aggregometry in detecting aspirin resistance and platelet dysfunction caused by the intake of aspirin in healthy volunteers (Marshall *et al.*, 1997). Marshall *et al.* (1997) also reported that the bleeding time measured using the Simplate™ method and PFA-100 closure time were in agreement with the inhibition of arachidonic acid-induced aggregation determined when aspirin is given to healthy volunteers. The same study suggested that PFA-100 demonstrated less false positive results than the bleeding time method, which is of the main concern when evaluating the effects of drugs on haemostasis. Another prospective investigation also suggested that PFA-100 is useful in the detection of platelet dysfunction, particularly during the preoperative period (Konrad *et al.*, 2006). However, more research is needed when comparing point-of-care testing such as PFA-100 to clinical outcomes related associated with poor aspirin response (Wong *et al.*, 2004).

The 96-well plate format of platelet aggregation has been shown to be a reliable method in determining aspirin-induced platelet inhibition, as described in our previous paper (Armstrong *et al.*, 2008). The ability to investigate TXB_2 levels using the same samples from agonist-induced aggregation in the 96-well plate format provides advantages in finding associations between platelet aggregation and platelet TXB_2 formation. For clinical testing, even though PFA-100 and other tests such as VerifyNow and multiplate electrical impedance aggregometry (MEA) are also reliable to test aspirin effects on platelets, pre-treatment values should be taken into account so that the risk of overestimation in the assessment of platelet inhibition by aspirin is eliminated (Can *et al.*, 2010).

Post occlusive forearm skin reactive hyperaemia is determined as increased skin blood flow to tissue following the release of short arterial occlusion (Tee *et al.*, 2004). Even though functional abnormalities of the microcirculation have been accepted as reflecting abnormalities in the pathogenesis of cardiovascular diseases, the exact mechanism of skin post-ischaemic hyperaemia responses are not fully established (Beinder *et al.*, 2001). Here the levels of COX prostanoids were investigated following reactive hyperaemia in healthy volunteers given parenteral diclofenac sodium. Measurement of PGE_2 production in response to LPS added to citrated blood samples indicates the induction of COX-2, whilst concurrent measurement of TXB_2 production in non-induced and calcium ionophore-induced citrated whole blood represents COX-1 activity before and after reactive hyperaemia. In parenteral administration of diclofenac, both TXA_2 and PGE_2 levels were decreased as a result from the non-selective COX inhibition of diclofenac. The opposite

actions of PGI_2 and TXA_2 on vascular systems are well studied; with the balance of those two being very crucial in the development of various thrombotic diseases (Yuhki *et al.*, 2011). TXA_2 is a potent vasoconstrictor which is produced mainly by platelets, whilst PGI_2 is a potent vasodilator and produced by vascular endothelial cells (Yuhki *et al.*, 2011). In a previous study performed in obese rats it was found that COX-1 derived prostanoids are responsible for vasoconstriction, but endothelial COX-2 is upregulated to increase production of vasodilator prostaglandins in insulin resistant obese rats (Sánchez *et al.*, 2010). Studies such as this demonstrate the importance of vasodilator prostanoids to the maintenance of normal basal vascular tone, which, however, can be limited by the vasoconstrictor products of COX (Campia *et al.*, 2002). Although PGE_2 is the most abundant prostanoids, its role in the skin reactive hyperaemia is not clearly observed in this study. Therefore, it is unclear whether PGE_2 or COX-2 is involved in the regulation of microcirculation. This study however, is limited as no measurements were made to look at 6-keto-prostaglandins F1α, a stable metabolite of prostacyclin. Diclofenac suppressed COX-1- and COX-2-dependent prostanoid production in citrated blood; i.e. decreased the production of TXA_2 and PGE_2. Consequently, the fact that TXA_2 is a vasoconstrictor will suggest an improvement of endothelial function in patients with risk factors for atherosclerosis for whom aspirin is given as part of therapeutic treatment (Campia *et al.*, 2002).

In conclusion, this study determined the use of 96-well plate format for monitoring platelet function in humans, and its potential usefulness as a routine laboratory platelet test. Further, this study shows that NSAIDS such as diclofenac is a platelet inhibitor, *in vitro* and *ex vivo* but not the COX-2 selective inhibitors, parecoxib and lumiracoxib, as shown *in vitro*. Diclofenac was also found to decrease vascular TXA_2 production, which is suppressed during post occlusive forearm skin reactive hyperaemia.

CHAPTER FOUR:

STUDY OF INFLUENCES OF 12-LIPOXYGENASE AND NADPH OXIDASE PATHWAYS ON PLATELET REACTIVITY

4.1 Introduction

Previous reports regarding 12-LOX function in platelets have been relatively limited and inconsistent. Irreversible platelet aggregation has been demonstrated to be independent of 12-LOX (Rao *et al.*, 1985), although the inhibitor used in this study was not specific to platelet 12-LOX. An important role for the 12-LOX pathway in platelets has been reported with 12(S)-HETE formation being increased following treatment of washed platelets with ATP, suggesting that the anti-aggregatory effects of ATP are due to enhance platelet 12(S)-HETE production (Dragan *et al.*, 1990). In the studies reported here the involvement of platelet 12-LOX in platelet aggregation and adhesion following stimulation by various agonists was examined. Two selective inhibitors of 12-LOX, baicalein (5, 6, 7-trihydroxyflavone) (Sekiya *et al.*, 1982) and CDC (cinnamyl-3, 4-dihydroxy-α-cyanocinnamade) (Kälvegren *et al.*, 2007) were used along with 12-LOX products, 12(S)-HPETE and 12(S)-HETE.

Platelet NADPH oxidase composed of gp91-phox and p22-phox, both are membrane-bound subunits that are responsible for the catalytic reaction of electron transfer from NADPH to oxygen molecule (Seno *et al.*, 2001). Besides that, NADPH oxidase also consists of regulatory subunits located intracellularly, small GTPases (Rac1 or Rac2) and cytosolic factors (p47-phox, p67-phox and p40-phox). Phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) -dependent phosphorylation induces the translocation of cytosolic factors and Rac to form NADPH oxidase complex, with gp91-phox and p22-phox thus

activating the catalytic reaction (Regier *et al.*, 1999). Recently, new homologues of the NADPH oxidase subunit, gp91-phox also known as Nox2, have been identified, so that the NADPH oxidase family currently consists of 7 members: Nox1, Nox2, Nox3, Nox4, Nox5, DUOX1 and DUOX2 (Brandes *et al.*, 2008).

It is well understood that phagocytes produce ROS through a respiratory burst for the purposes of bacterial killing. ROS formation in platelets may play a different role, as suggested by previous evidence of aggregation-induced ROS production. Thus, as well as investigating the LOX pathway, we have studied the role of NADPH oxidase in platelets. Diphenylene iodonium (DPI) and apocynin, often used as a classic NADPH oxidase inhibitors, were used in this study however the specificity of these inhibitors are unclear (Brandes *et al.*, 2008). This is due to a recent report suggesting that apocynin function as an antioxidant in endothelial cells and vascular smooth muscle cells, thus its inhibitory actions on NADPH oxidase is debatable (Heumüller *et al.*, 2008).

4.2 Methodology

To study the biological function of 12-LOX in platelets both well-known inhibitors of 12-LOX, baicalein (Sekiya *et al.*, 1982) and CDC (Kälvegren *et al.*, 2007) were used. Citrated PRP were used throughout this study unless stated otherwise. The concentration of baicalein used was 10µM except when concentrations ranging from 0.001-100µM were used to establish concentration-dependent effects. In the experiment where baicalein was co-incubated with aspirin, 30µM aspirin was used. For CDC, a concentration of 300µM was chosen to investigate its effects on platelet responses to agonist stimulation. Preparation of platelet agonists and combined agonists was as described in previous chapter. In all experiments, incubation with inhibitors was for 30 min before determination of platelet aggregation and adhesion by the 96-well plate method described in previous chapter. In the study of 12-LOX products on platelet functions, 20pg/ml 12(S)-HPETE and 12(S)-HETE were used, with extra care being taken during preparation as these peroxides are easily oxidised and degraded. In contrast with 12-LOX inhibitors, any incubation involving 12(S)-HPETE or 12(S)-HETE was for 2 min because of the short half-lives of these peroxides.

300µM DPI and 1mM apocynin (Begonja *et al.*, 2005) were used as NADPH oxidase inhibitors in this study. However, for study of the concentration dependent effects of DPI a range from 50-300µM was used. Following 30 min incubation with NADPH oxidase

inhibitors, platelet aggregation and adhesion studies were performed as previously described. Statistical analyses were performed as appropriate.

4.3 Results

4.3.1 Effects of 12-Lipoxygenase Inhibitors, Baicalein and Cinnamyl-3,4-Dihydroxy-Cyanocinnamate (CDC) on Platelet Activation by Various Agonists

Baicalein decreased platelet aggregation in adrenaline and AA-stimulated platelet and weakly reduced ADP- and ristocetin–induced aggregation whilst no effects were against the other agonists (Figure 4.1). For instance, controls aggregation when induced by 0.3, 1.0 and 1.3mM AA were 25±24%, 63±22% and 63±14% were reduced to 21±23%, 17±22% and 41±22%, respectively, in the presence of baicalein. Aggregations induced by 1µM and 10µM adrenaline were decreased from 33±15% and 35±12% to 13±6% and 24±7% by baicalein. Platelet adhesion was also affected by baicalein, as adhesions induced by AA, adrenaline and ADP were reduced (Figure 4.2). For example, when incubated with baicalein, adhesion induced by 1µM and 10µM adrenaline was decreased to 7±3% and 13±5% from 15±7% and 22±8% in controls.

CDC had little effect upon platelet aggregation and adhesion when PRP prepared from citrated blood was used, as shown in Figure 4.3 and Figure 4.4. There was slightly reduced platelet aggregation to the middle concentrations of collagen but no effect on other agonists' stimulation, with decreased of platelet adhesion only being demonstrated in ristocetin-treated platelets. For instance, control adhesions following 1 and 2mg/ml ristocetin were 10±8% and 15±6%, which were reduced to 0% and 12±3% in the presence

of CDC. As citrated PRP was less sensitive to CDC, heparinised PRP was used to investigate further the effects of CDC upon platelet activation. In these experiments platelet aggregations induced by collagen, adrenaline and ristocetin were slightly decreased whilst no changes were seen in other agonist-induced aggregations (Figure 4.5). Moreover, slight reduction in platelet adhesion following stimulation with ADP, collagen and ristocetin was observed in CDC-treated platelets, as shown in Figure 4.6. For instance, ADP at 10 and 30μM stimulated adhesion to 26±11% and 32±14% which were decreased to 22±9% and 27±12% with CDC. Although the inhibitory effects of baicalein and CDC are varied and differed with the agonists used to activate the platelets, there was evidence that the platelet 12-LOX pathway is involved during activation of platelets by AA, adrenaline, ADP, collagen and ristocetin but not by TRAP-6 and U46619. The variability of inhibition by both 12-LOX inhibitors suggested that the action is dependent on specific agonists where primary platelet activation is involved.

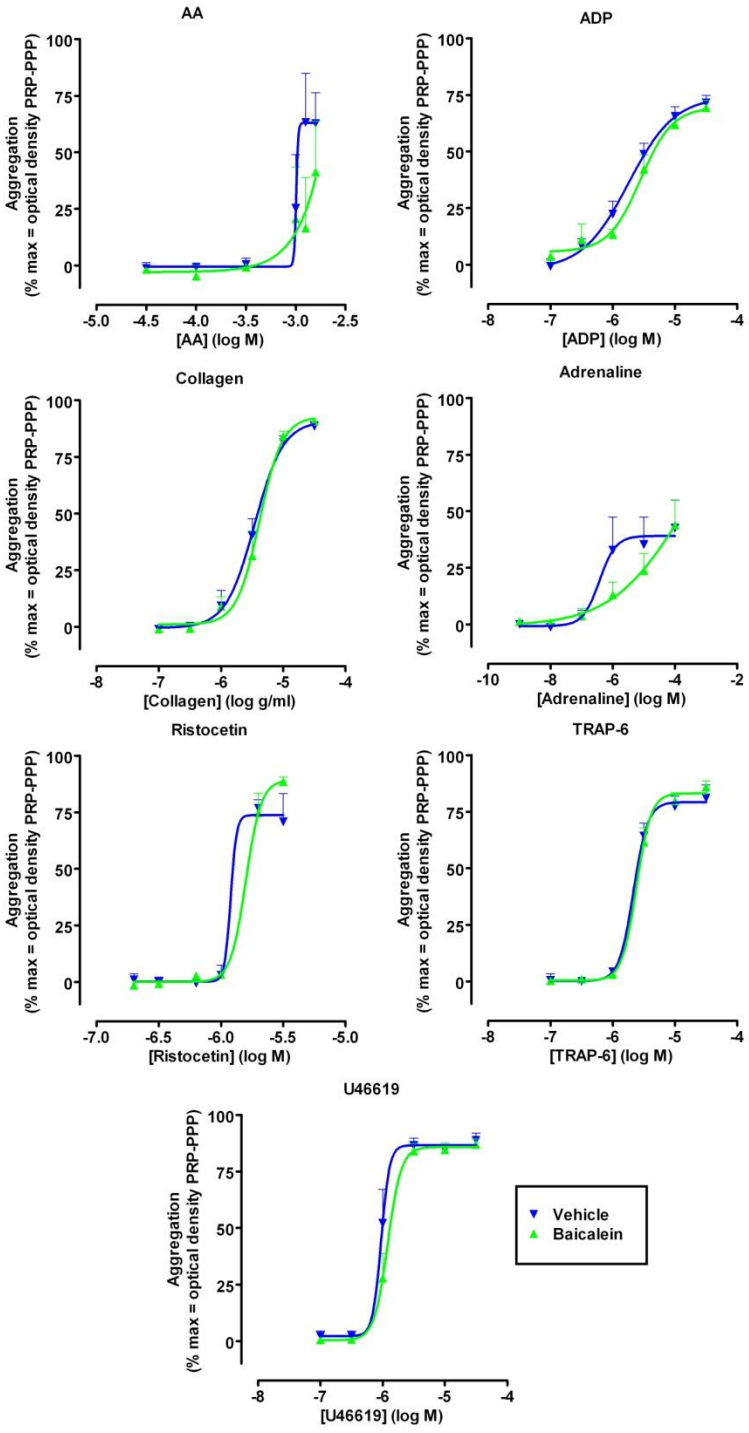


Figure 4.1: *Inhibitory effects of baicalein on platelet aggregation in response to various agonists.* PRP was incubated with 10µM baicalein before agonist-stimulated platelet aggregation was measured in 96-well plates. Each data point represents mean ± S.E.M. (n=4).

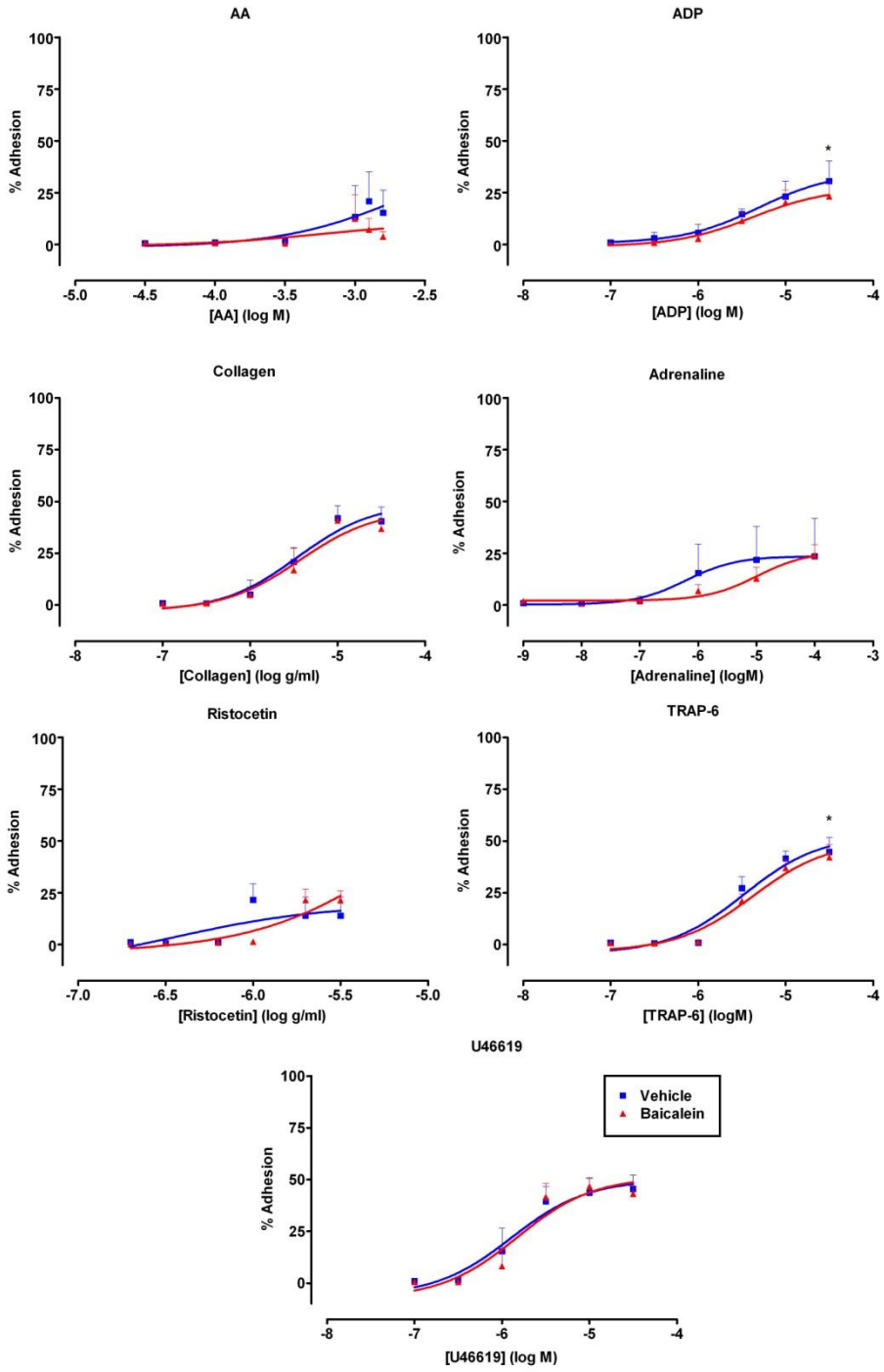


Figure 4.2: *Inhibitory effects of baicalein on platelet adhesion in response to various agonists.* Each data point represents mean ± S.E.M. (n=4). * = P<0.05 as determined by two-way ANOVA.

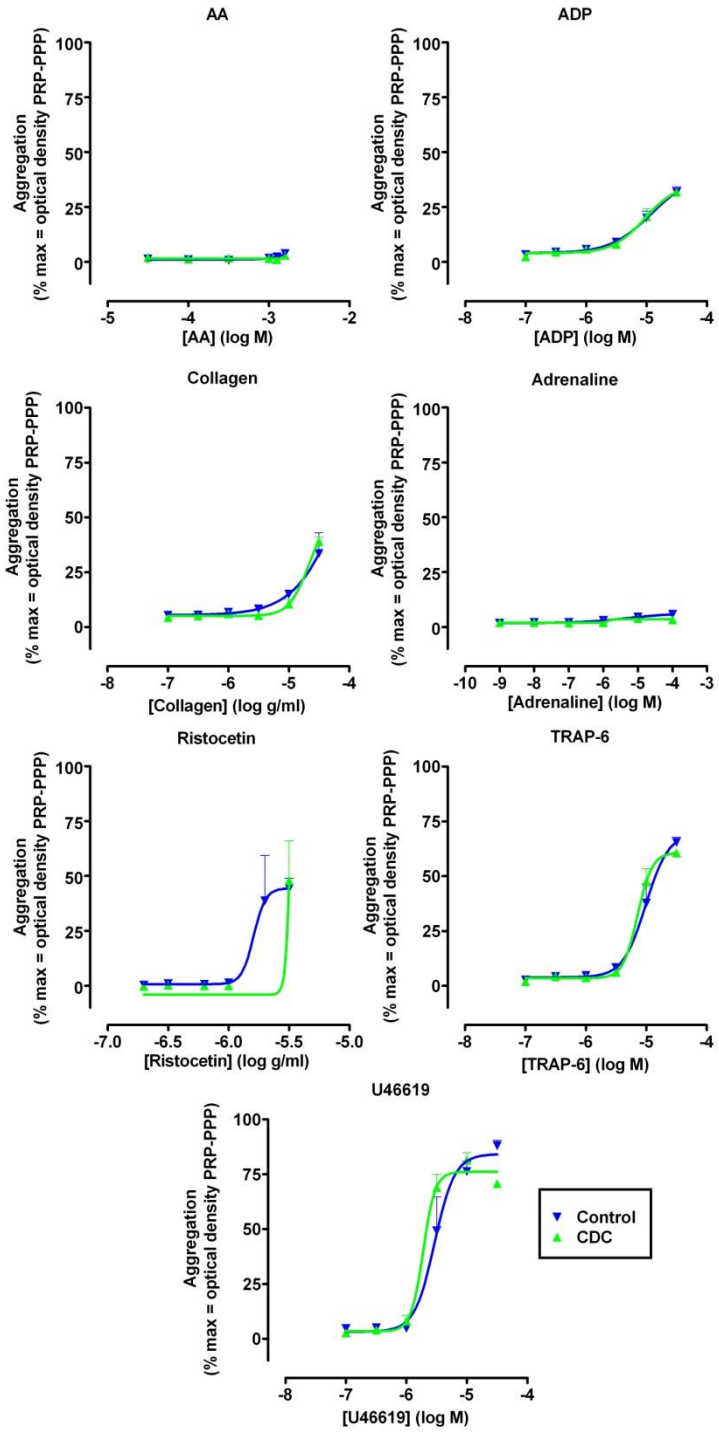


Figure 4.3: *Inhibitory effects of CDC on platelet aggregation in response to various agonists*. Citrated PRP was incubated with 300µM CDC before agonist-stimulated platelet aggregation was measured in 96-well plate. Each data point represents mean ± S.E.M. (n=4).

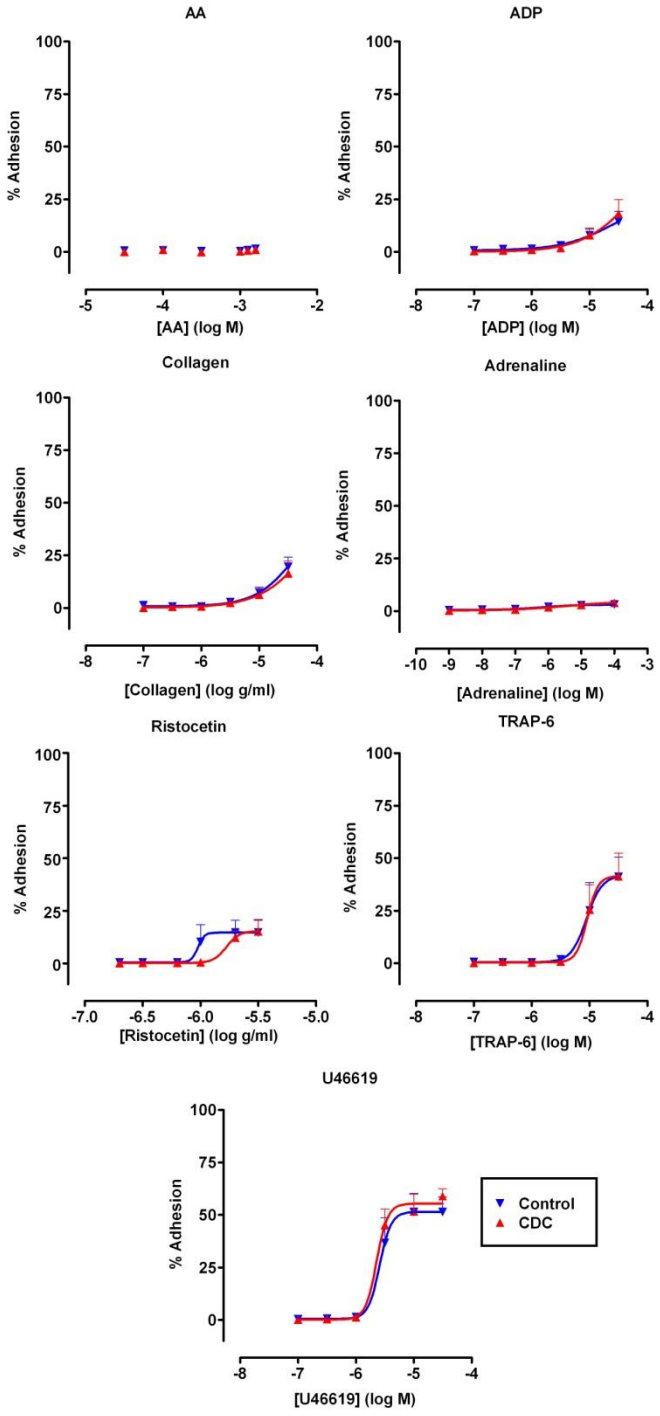


Figure 4.4: *Inhibitory effects of 300µM CDC on platelet adhesion in citrated PRP in response to various agonists.* Each data point represents mean ± S.E.M. (n=4).

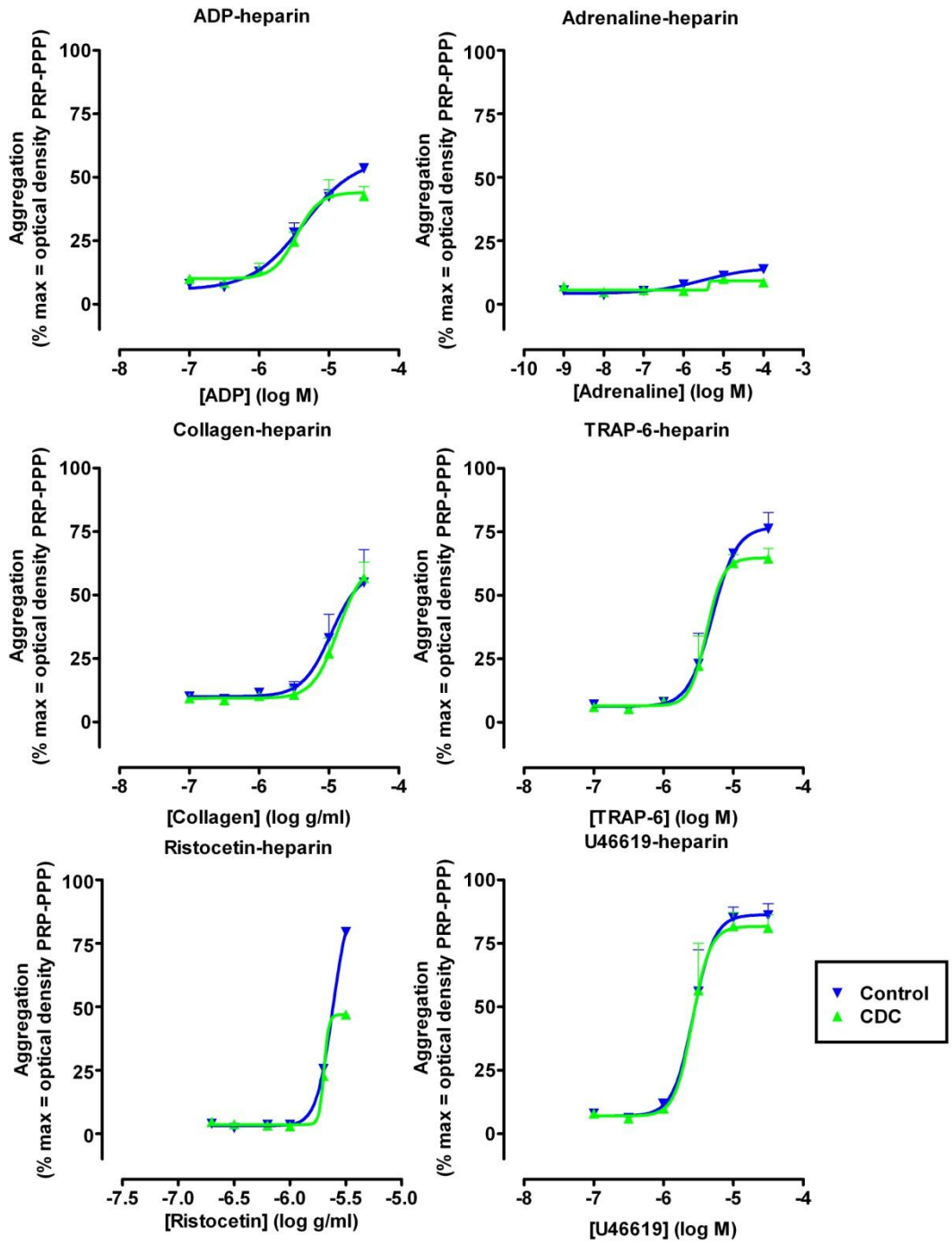


Figure 4.5: *Inhibitory effects of CDC on platelet aggregation in response to various agonists*. Heparinised PRP was incubated with 300µM CDC before agonist-stimulated platelet aggregation was measured in 96-well plate. Each data point represents mean ± S.E.M. (n=4).

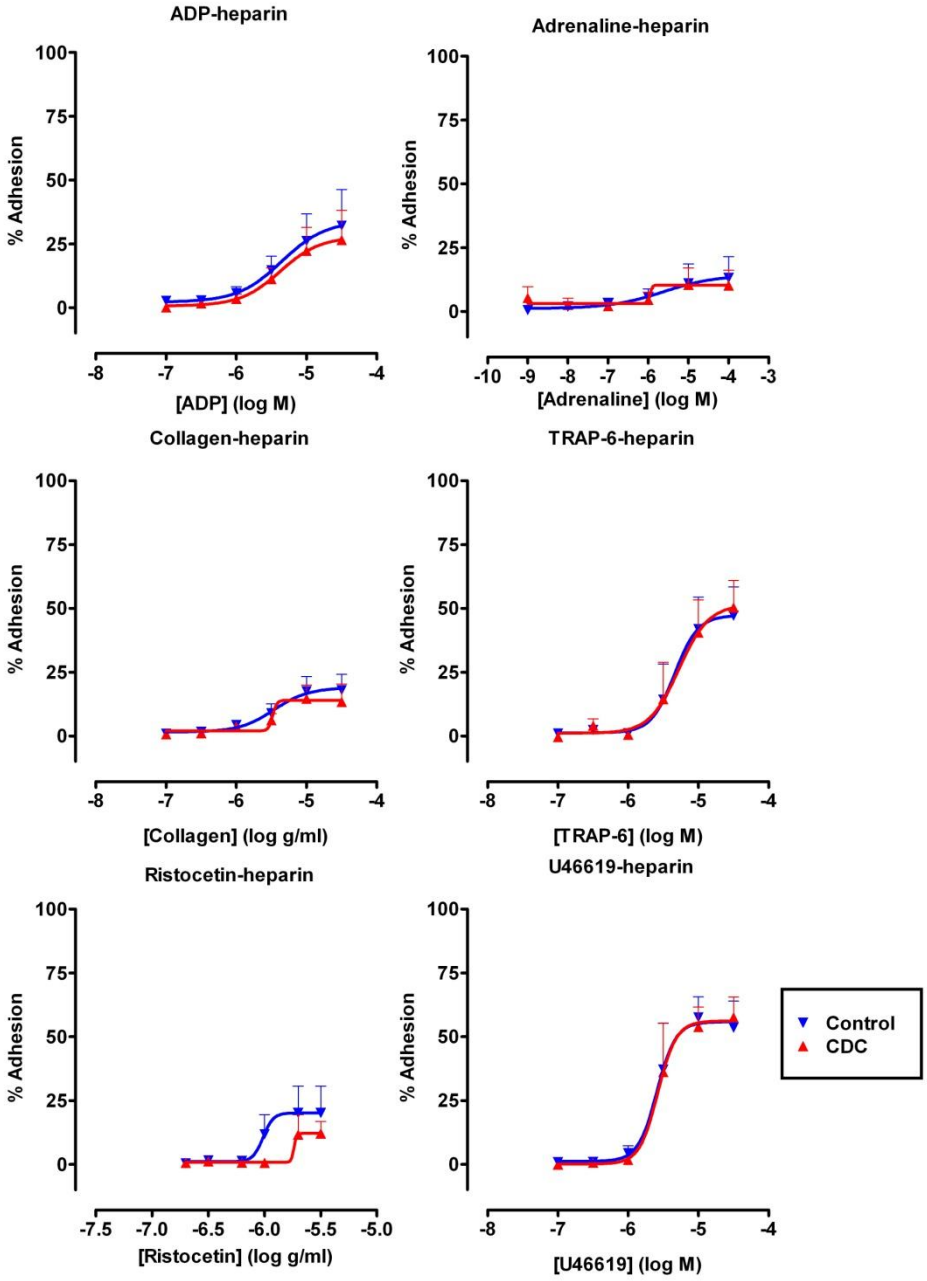


Figure 4.6: *Inhibitory effects of 300μM CDC on platelet adhesion in citrated PRP in response to various agonists.* Each data point represents mean ± S.E.M. (n=4).

4.3.2 Effects of Baicalein on Platelet Activation in Response to Combination of Collagen and Adrenaline

PRP was incubated with baicalein, 0.001-100μM, before introduction of either single or combined agonists. As shown in Figure 4.7, platelet aggregation increased with the use of combined agonists as compared to single agonists as expected. In the presence of baicalein, there was a concentration-dependent reduction platelet aggregation and adhesion. For instance, in PRP activated by combined collagen and 0.1μM adrenaline, control aggregation was 41±7% decreased to 34±8%, 31±6% and 29±7% for baicalein at 0.01, 0.1 and 1μM baicalein, respectively. The decrease of platelet adhesion was most clearly affected in single agonist and combined agonists' experiments when adrenaline was used at 0.01μM. This data suggests that when platelets are activated by low concentrations of collagen or adrenaline, 12-LOX is particularly involved.

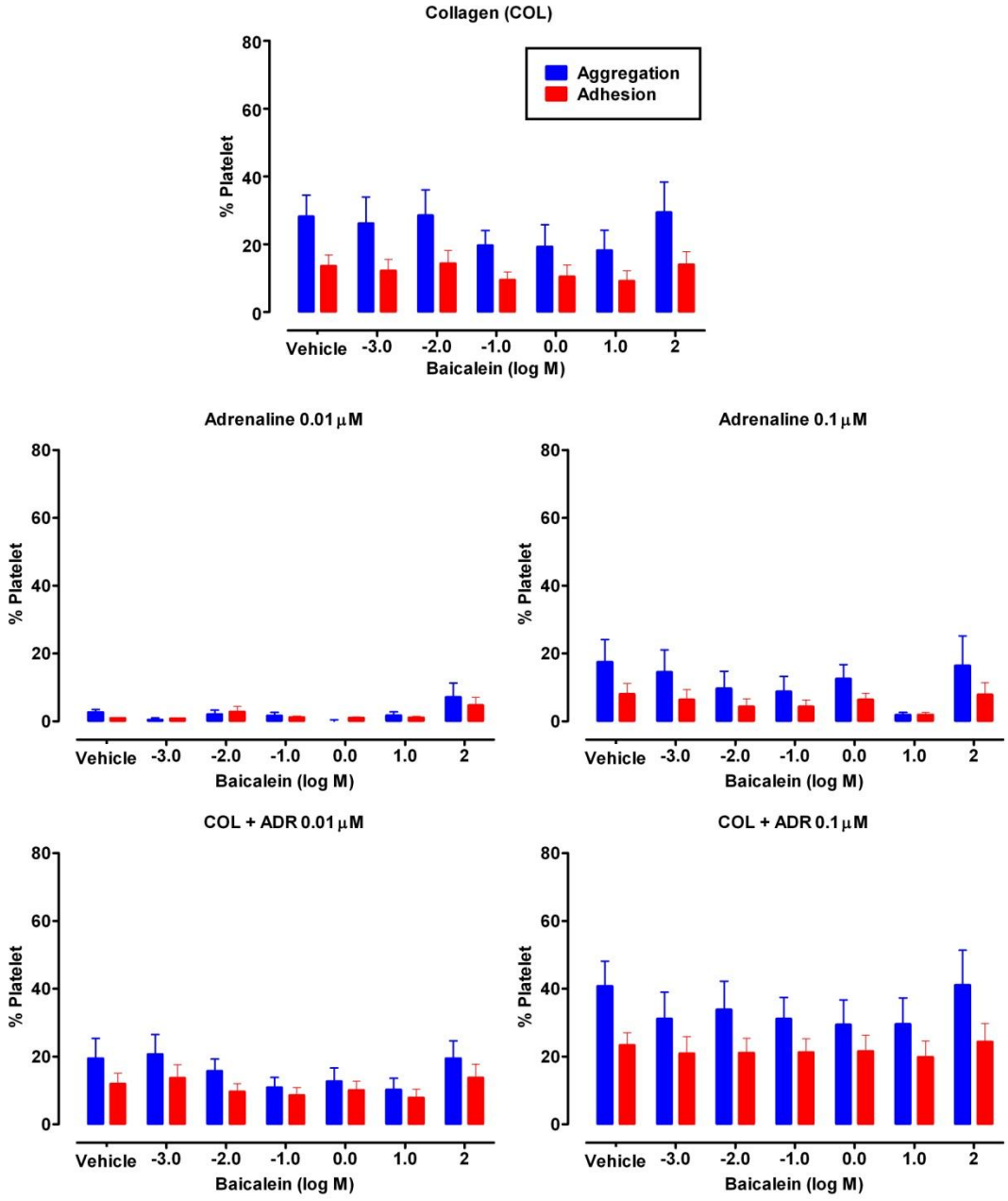


Figure 4.7: *Concentration-dependent effects of baicalein on platelet aggregation and adhesion induced by combined agonists.* PRP was incubated with various concentrations of baicalein before addition of combination of collagen (1µg/ml) and adrenaline (ADR: 0.1 and 0.01µM). Each data point represents mean ± S.E.M., n=4.

4.3.3 Effects of Combination of Aspirin and Baicalein on Platelet Activation in Response to Various Platelet Agonists

Aggregation in response to most agonists was not affected by combination of aspirin and baicalein, except for that to AA (Figure 4.8). In the presence of aspirin alone, aggregations induced by AA at 0.3, 1.0 and 1.3mM were 17±11%, 27±14% and 32±1%. However, when co-incubated with baicalein, these were reduced to 0%, 7.0±2% and 27±8%. Similar observations were demonstrated for platelet adhesion in which only AA and ristocetin-induced platelet adhesion were decreased, as shown in Figure 4.9. For instance, percentage of aspirin-incubated platelet adhesion was 7±5% and 12±5% at 0.3 and 1.0mM AA, which were reduced to 1±1% and 2±1% in the presence of baicalein. Ristocetin-induced adhesion was decreased to 1±1% in the presence of both aspirin and baicalein as compared to 22±5% in the presence of aspirin alone. This suggested that when COX pathway is inhibited, the platelet may shift to become more dependent on the 12-LOX pathway.

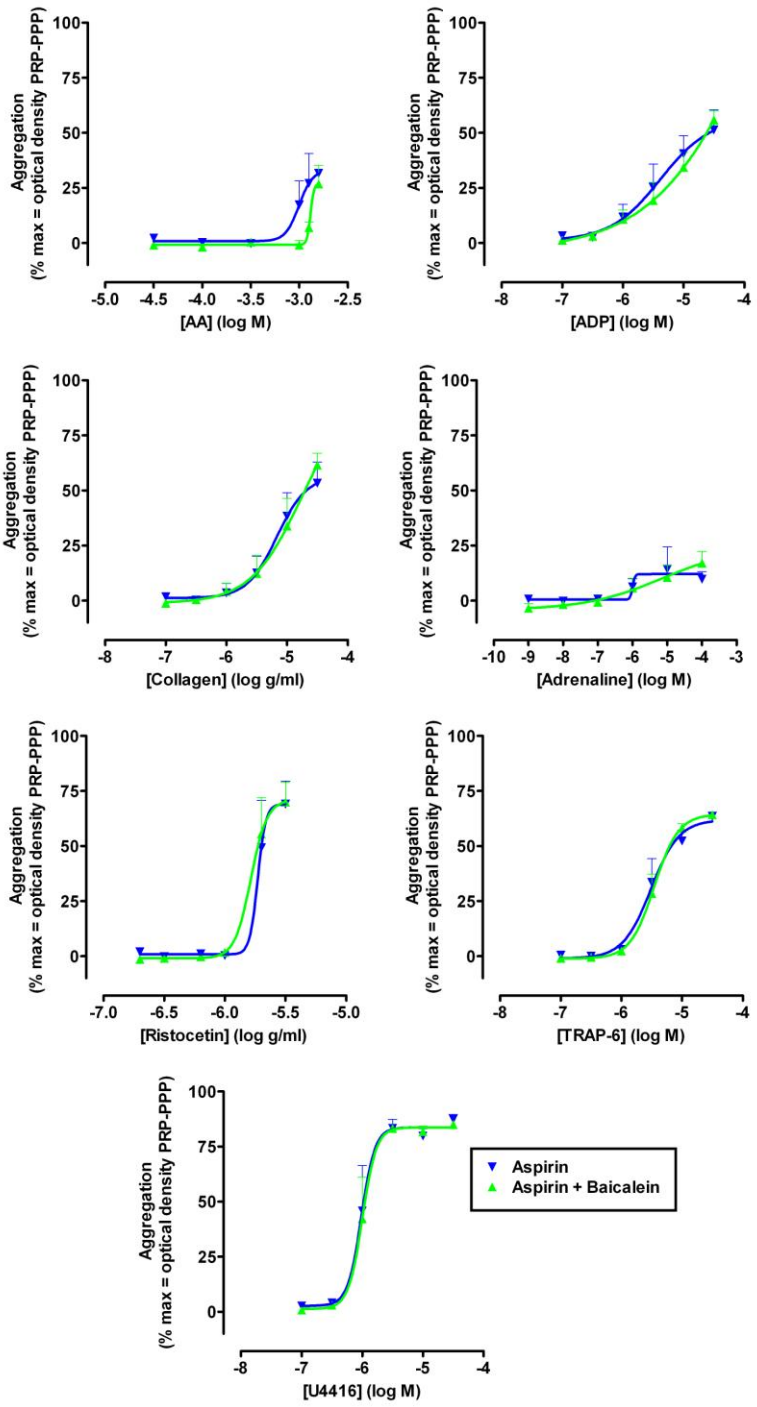


Figure 4.8: *Effects of combination of aspirin and baicalein on platelet aggregation in response to various agonists*. PRP was incubated with 30µM aspirin with or without combination of 10µM baicalein before agonist-stimulated platelet aggregation was measured in 96-well plates. Each data point represents mean ± S.E.M. (n=4).

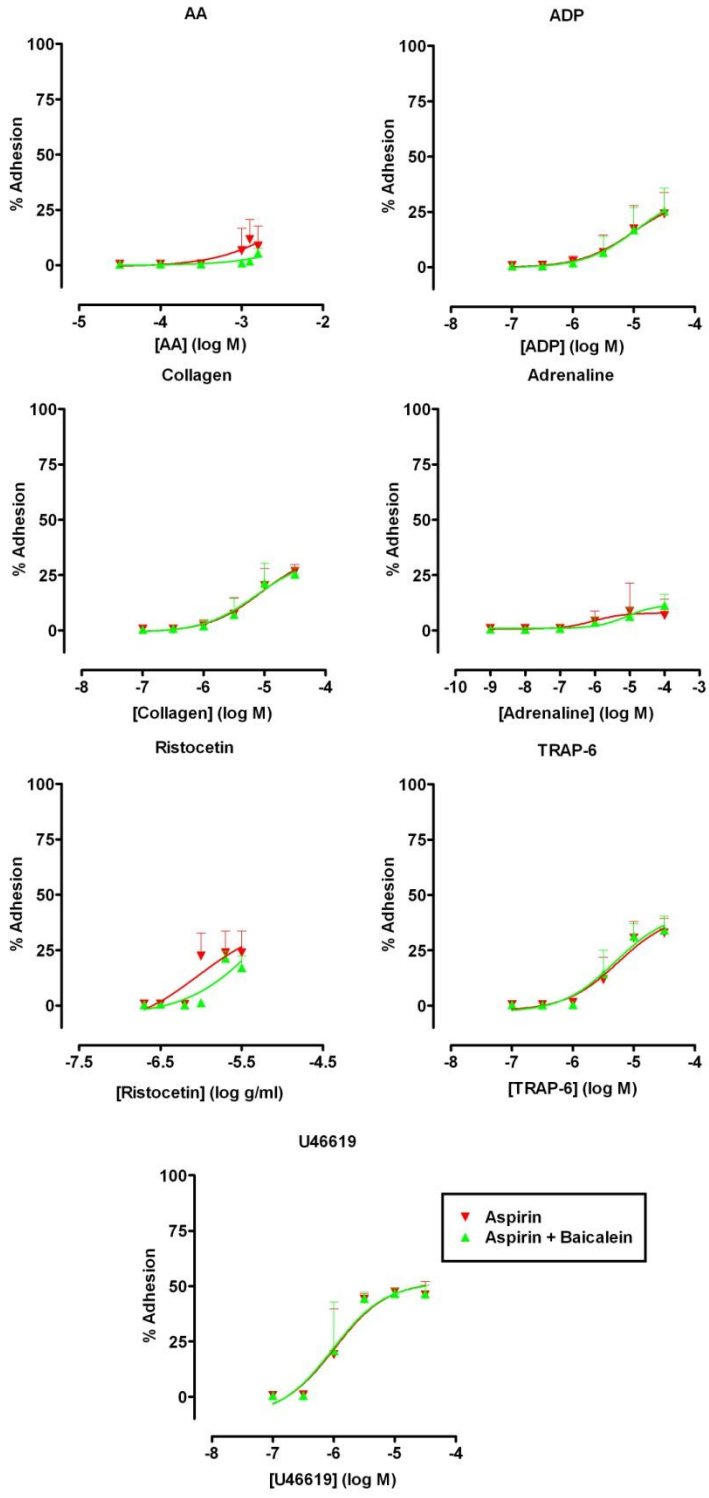


Figure 4.9: *Effects on platelet adhesion of combination of aspirin and baicalein*. Each data point represents mean ± S.E.M. (n=4).

4.3.4 Effects 12(S)-HETE on Platelet Activation by Various Agonists

Addition of exogenous 12(S)-HETE increased platelet aggregation induced adrenaline and ristocetin but not that induced by any other agonists (Figure 4.10). For instance, aggregation induced by 10 and 100μM adrenaline in control condition was 31±16% and 34±13% which were increased to 36±14% and 58±9% in the presence of 12(S)-HETE. As shown in Figure 4.11, adhesion in response to AA, ADP, ristocetin and adrenaline were enhanced by exogenous 12(S)-HETE, whereas adhesion to other agonists was little affected. For example, for adrenaline-stimulated adhesion, controls were 16±10% and 14±9% at 10 and 100μM agonist concentrations which were increased to 23±9% and 33±7% in the presence of exogenous 12(S)-HETE.

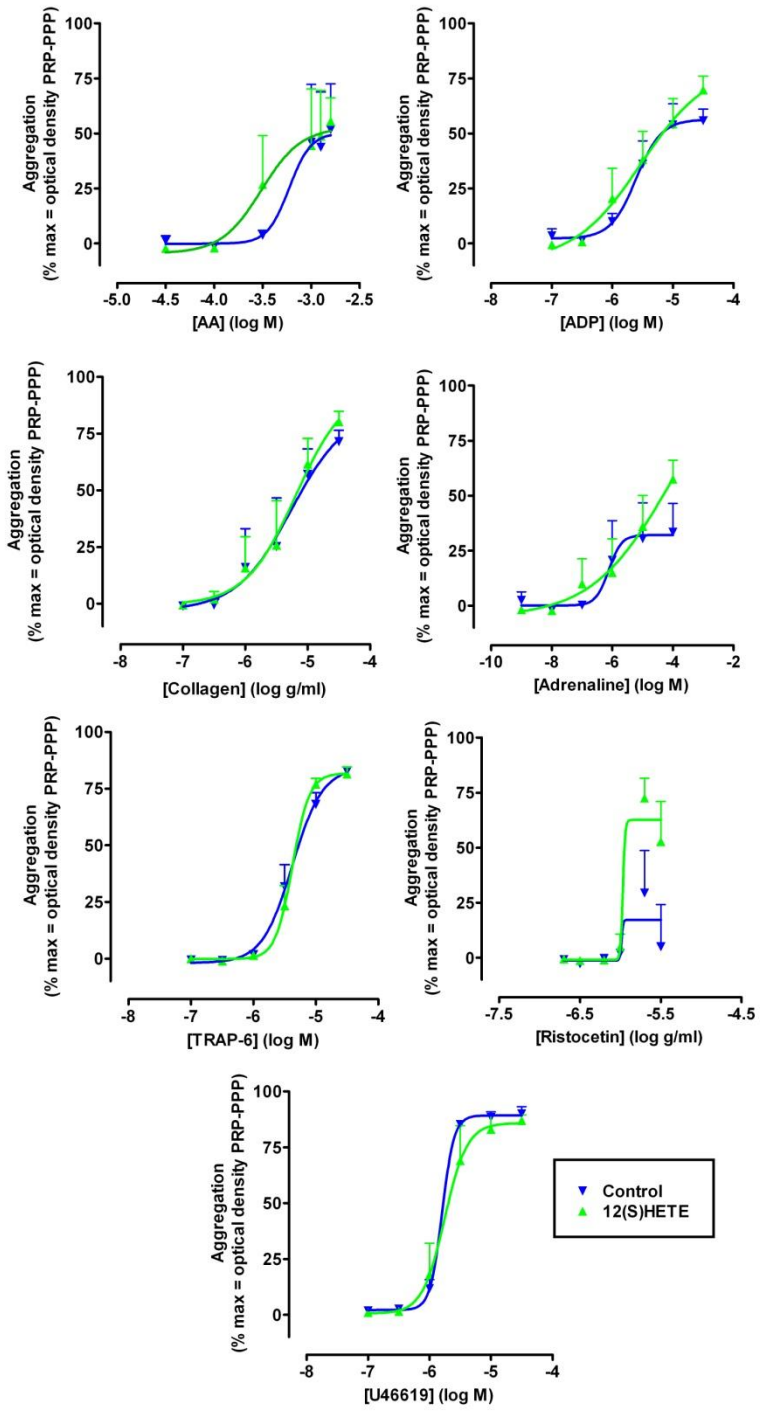


Figure 4.10: *Effects of 12(S)-HETE on platelet aggregation stimulated by various agonists*. PRP was incubated with 20pg/ml 12(S)-HETE before agonist-stimulated platelet aggregation was measured in 96-well plates. Each data point represents mean ± S.E.M. (n=4).

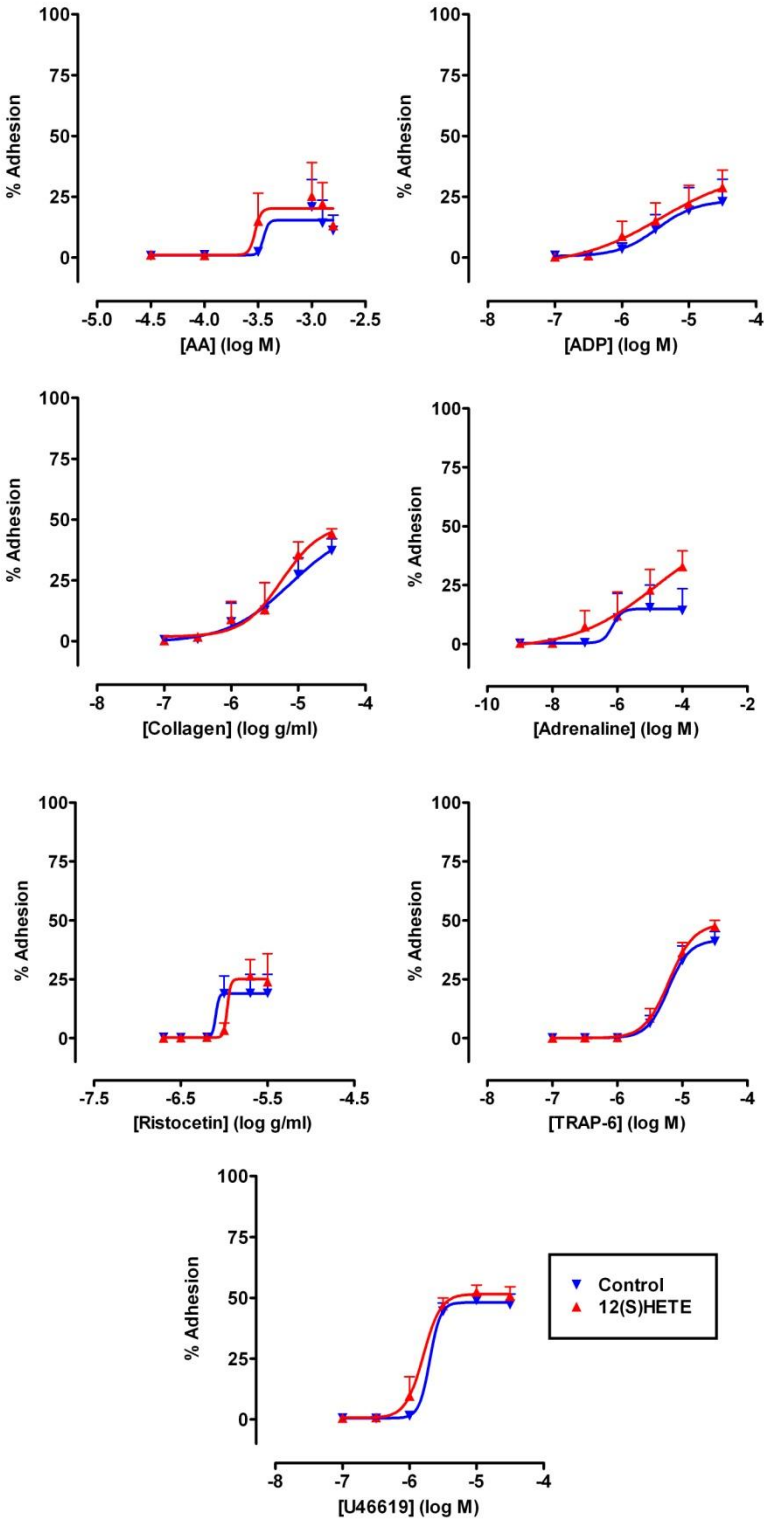


Figure 4.11: *Effects on platelet adhesion of 12(S)-HETE stimulated by various agonists*. Each data point represents mean ± S.E.M. (n=4).

4.3.5 Effects of 12(S)-HPETE on Platelet Activation by Various Agonists

12(S)-HPETE did not affect platelet aggregation in response to most agonists except AA, for which aggregation was reduced (Figure 4.12). For instance, platelet aggregations in response to 1.0 and 1.3mM AA were 21±17% and 49±13%, which were decreased to 4±1% and 17±4%, respectively. Platelet adhesion showed less effect of 12(S)-HPETE against most agonists with the exception of AA and ristocetin for which adhesion was reduced (Figure 4.13).

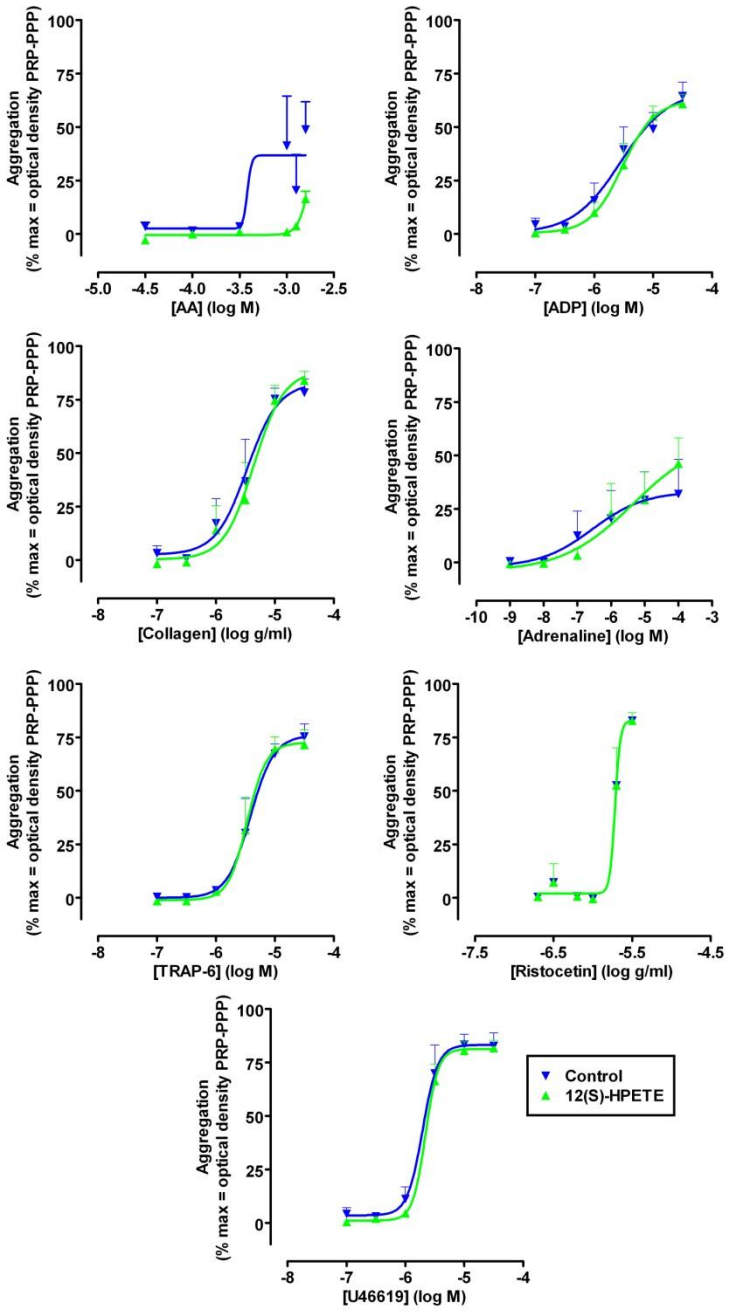


Figure 4.12: *Effects of 12(S)-HPETE on platelet aggregation stimulated by various agonists*. PRP was incubated with 20pg/ml 12(S)-HPETE before agonist-stimulated platelet aggregation was measured in 96-well plates. Each data point represents mean ± S.E.M. (n=4).

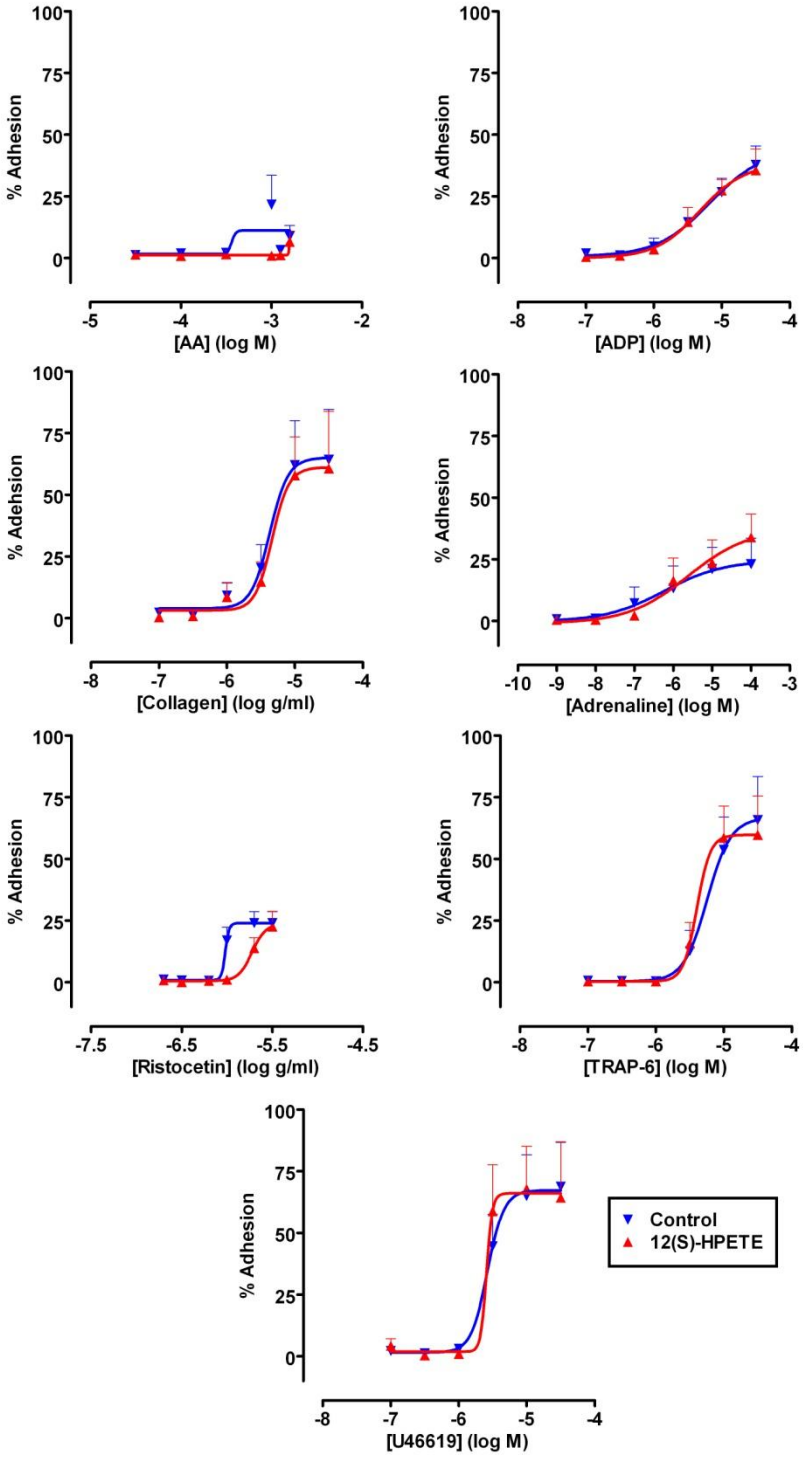


Figure 4.13: *Effects on platelet adhesion of 12(S)-HPETE stimulated by various agonists*. Each data point represents mean ± S.E.M. (n=4).

4.3.6 Effects of CDC and 12(S)-HETE on Platelet Activation Stimulated by Various Agonists

As shown in Figure 4.14, there was not much effect of exogenous 12(S)-HETE on platelet aggregation and adhesion. However, ristocetin-induced platelet adhesion was reduced in the presence of exogenous 12(S)-HETE (Figure 4.15). For example, platelet adhesions when 12-LOX was blocked were 8±3% and 18±9% in response to 1.0 and 2.0 mg/ml ristocetin and these were reduced to 1±1% and 10±5% in the presence of exogenous 12(S)-HETE.

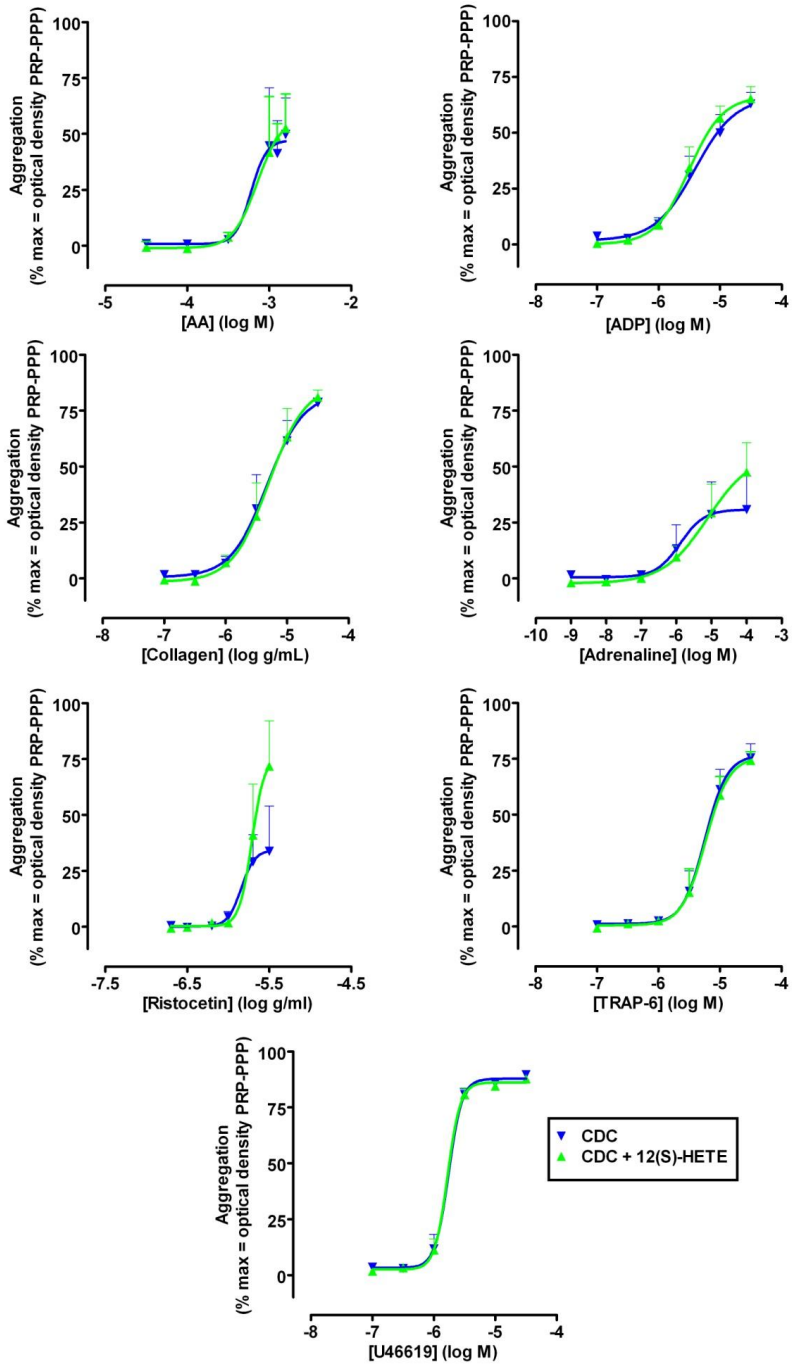


Figure 4.14: *Effects of 12(S)-HETE on CDC pre-treated platelet aggregation stimulated by various agonists*. PRP was incubated with 300µM CDC and then 20pg/ml 12(S)-HETE before agonist-stimulated platelet aggregation was measured in 96-well plates. Each data point represents mean ± S.E.M. (n=4).

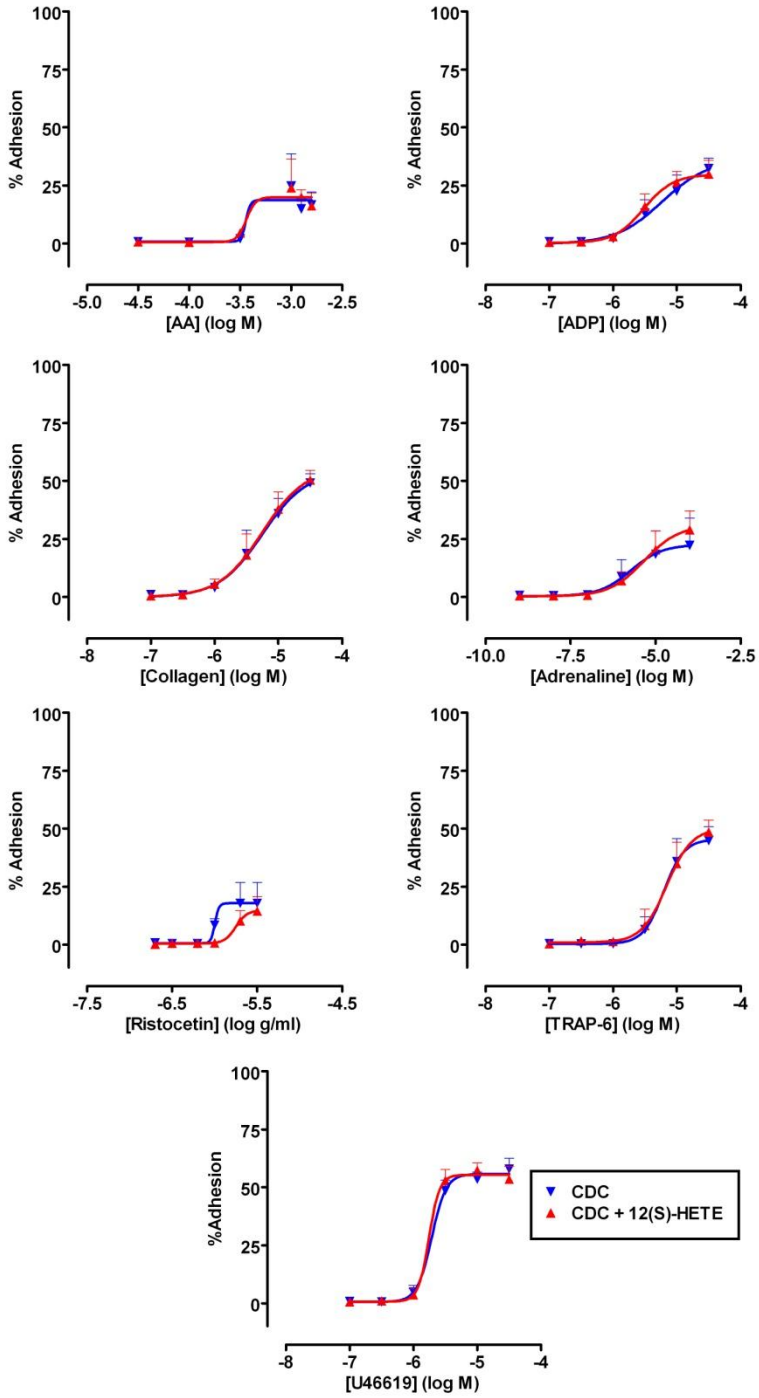


Figure 4.15: *Effects on platelet adhesion of combined CDC and 12(S)-HETE*. Each data point represents mean ± S.E.M. (n=4).

4.3.7 Effects of CDC and 12(S)-HETE on Platelet Activation Stimulated By Combination of Collagen and Adrenaline

As shown in Figure 4.16 and Figure 4.17, CDC reduced activation of platelets by single and combined agonists. For instance, collagen-induced aggregation in control, 54±2%, was reduced to 48±10, 39±6 and 35±6% by 0.1, 1 and 10μM CDC, respectively. Interestingly, platelet aggregation was increased by the addition of exogenous 12(S)-HETE in the presence of 10μM CDC, the response being 48±7%. The pattern of increase platelet aggregation following addition of exogenous 12(S)-HETE was also observed in PRP activated by 0.1μM adrenaline and by the combination of collagen with both adrenaline concentrations. Platelet adhesion was less affected by CDC except for that to 0.1 μM adrenaline which was decreased.

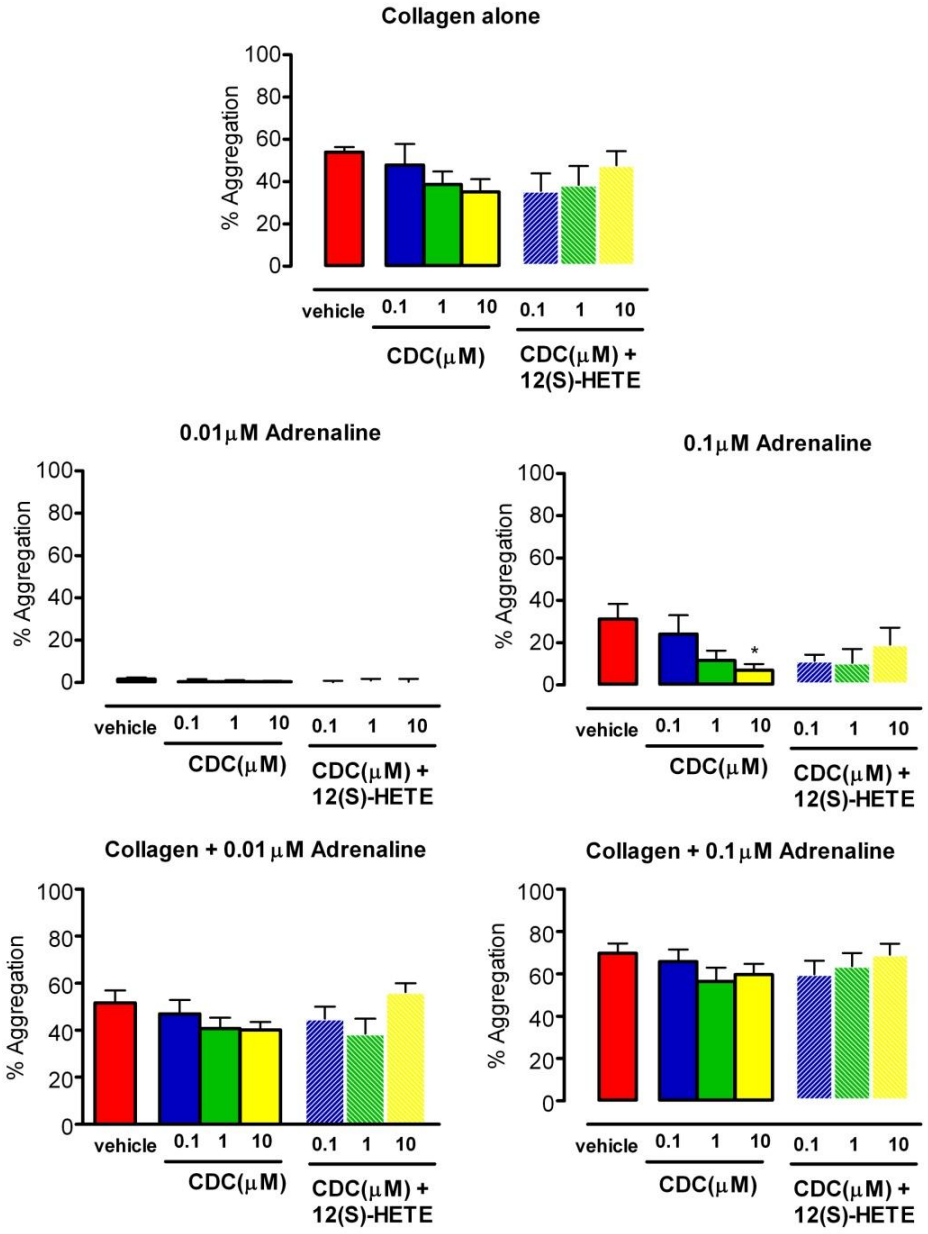


Figure 4.16: *Effects of CDC (0.1-10μM) alone or in the presence of 20pg/ml 12(S)-HETE on platelet adhesion stimulated by collagen, adrenaline or combination of both agonists*. Data shown are mean ± S.E.M. from duplicate reading of PRP from 4 different individuals. *P<0.05 when compared to vehicle as analyzed by one-way ANOVA.

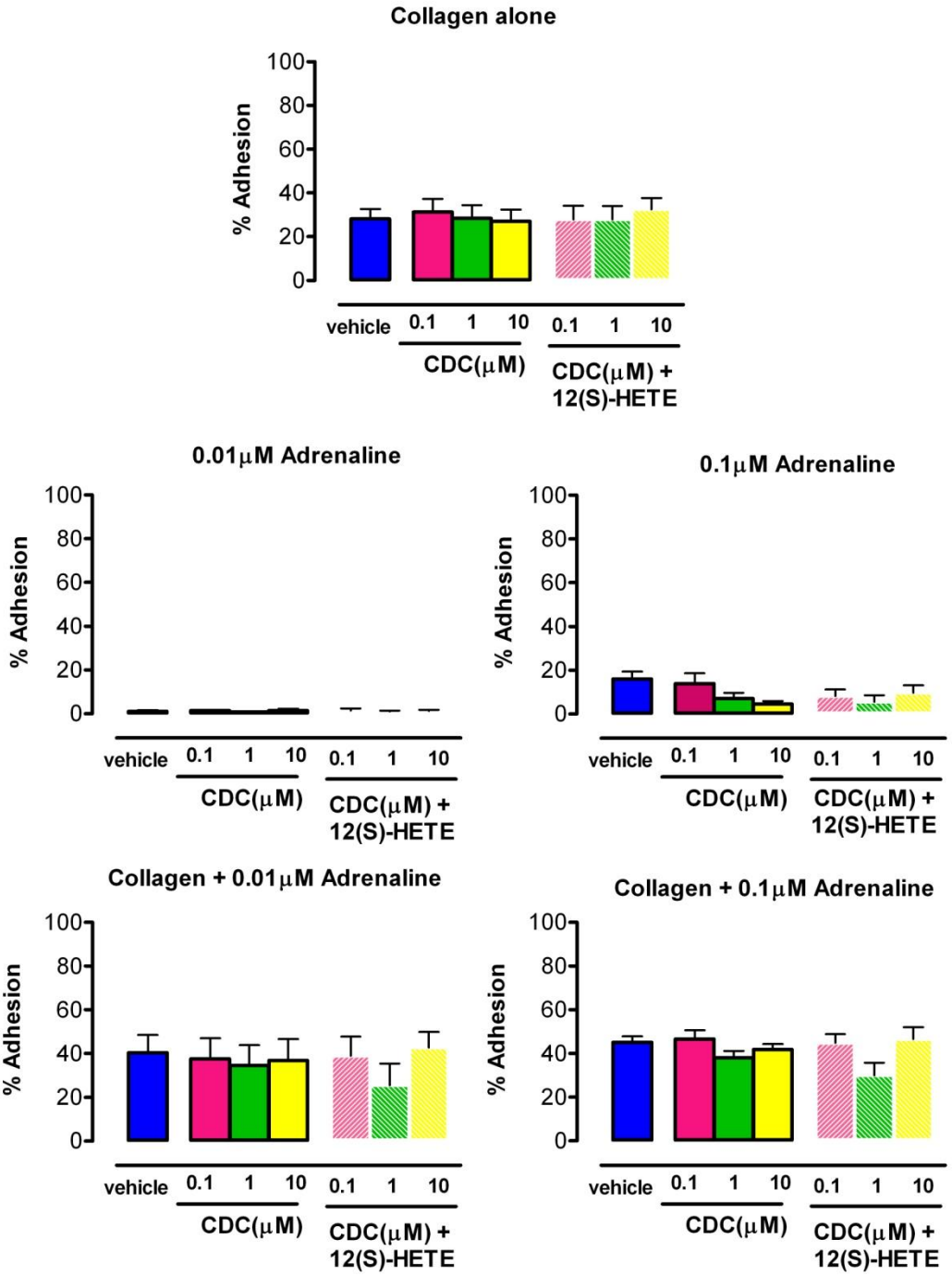


Figure 4.17: *Effects of CDC (0.1-10μM) alone or in the presence of 20pg/ml 12(S)-HETE on platelet adhesion stimulated by collagen, adrenaline or combination of both agonists*. Data shown are mean ± S.E.M. from duplicate reading of PRP from 4 different individuals.

4.3.8 Effects of Diphenylene Iodonium Chloride (DPI), a NADPH Oxidase Inhibitor, on Platelet Activation by Various Agonists

DPI at 300μM attenuated platelet aggregation induced by ADP, collagen and adrenaline with lesser effects on TRAP-6–induced platelet activation, whilst aggregations induced by other agonists were not affected (Figure 4.18). For instance, aggregation induced by 10 (23±9%) and 30μM (40±7%) ADP was reduced to 15±8% and 24±7% respectively. Similarly, collagen-induced aggregation at 10 (37±16%) and 30μM (38±23%) was reduced to 21±13% and 19±13%, respectively. With 100μM adrenaline, platelet aggregation was reduced to 11±7% from 23±8%.

DPI had greater effects against platelet adhesion, where DPI clearly reduced that induced by ADP, adrenaline, TRAP-6, ristocetin and slightly collagen (Figure 4.19). However, the inhibition of platelet adhesion to adrenaline and ristocetin were best seen at low agonists' concentrations. For instance, at 0.1 and 1μM adrenaline, 11±4% and 21±9% platelet adhesions were reduced to 3±2% and 7±3%, respectively. Similar observations were made for platelet adhesions stimulated by 1.0 (28±4%) and 2.0mg/mL (37±6%) ristocetin whihch were reduced to 2±1% and 24±6%, respectively. Based on these results, three agonists were chosen to investigate the inhibitory effects of DPI concentrations ranging from 50-300μM. For all agonists tested, collagen, ADP and TRAP-6, DPI inhibited both platelet adhesion and aggregation in a concentration-dependent manner, as shown in Figure 4.20.

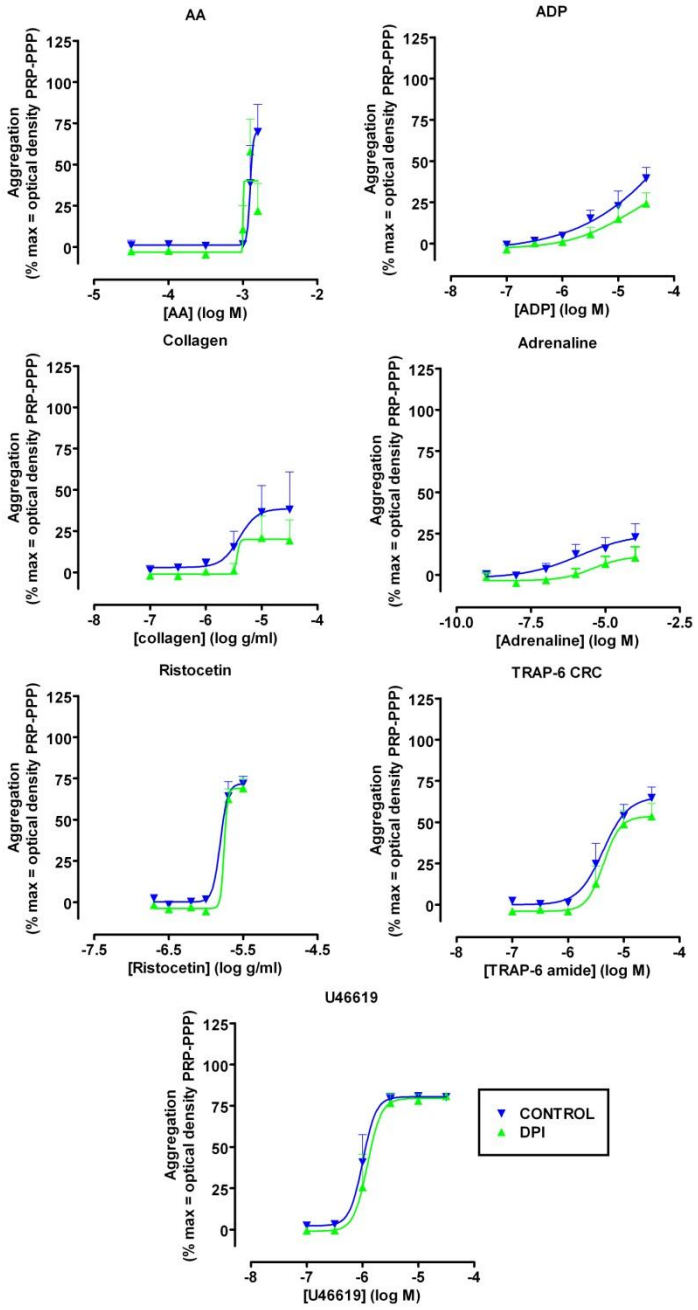


Figure 4.18: *Effects of DPI, a NADPH oxidase inhibitor on platelet aggregation stimulated by various agonists*. PRP was incubated with 300µM DPI before agonist-stimulated platelet aggregation was measured in 96-well plates. Each data point represents mean ± S.E.M. (n=4).

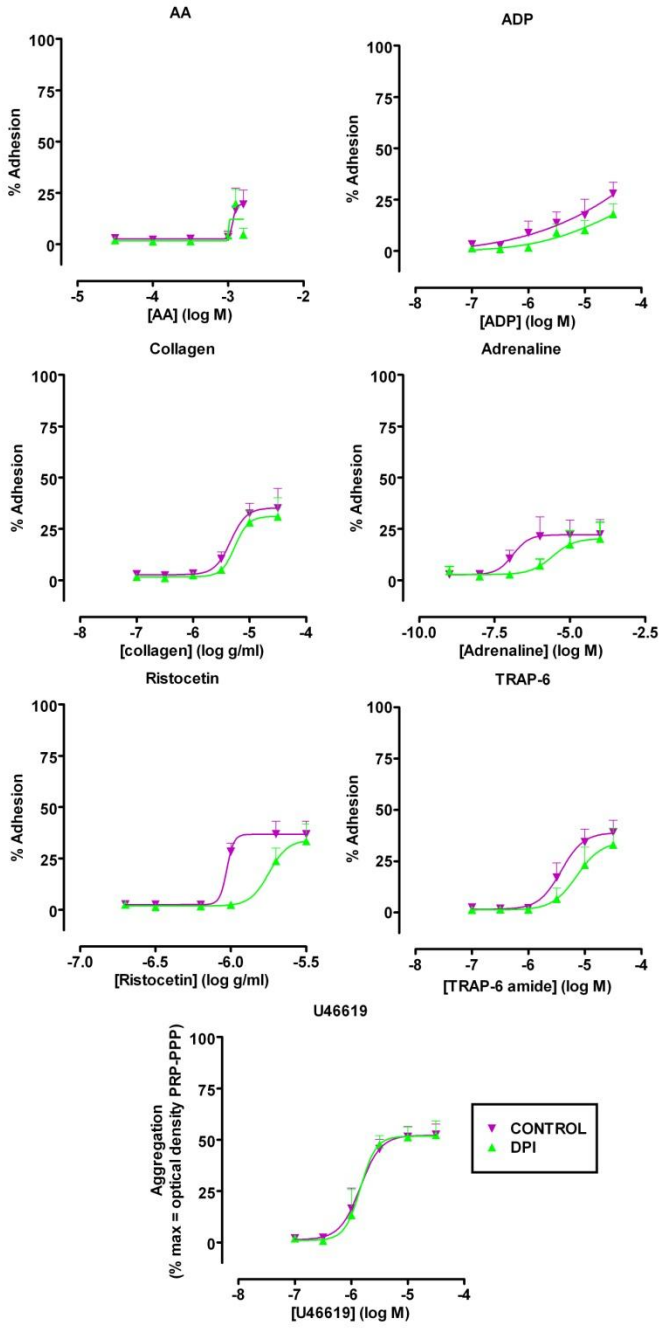


Figure 4.19: *Effects of DPI, a NADPH oxidase inhibitor on platelet adhesion stimulated by various agonists*. Each data point represents mean ± S.E.M. (n=4).

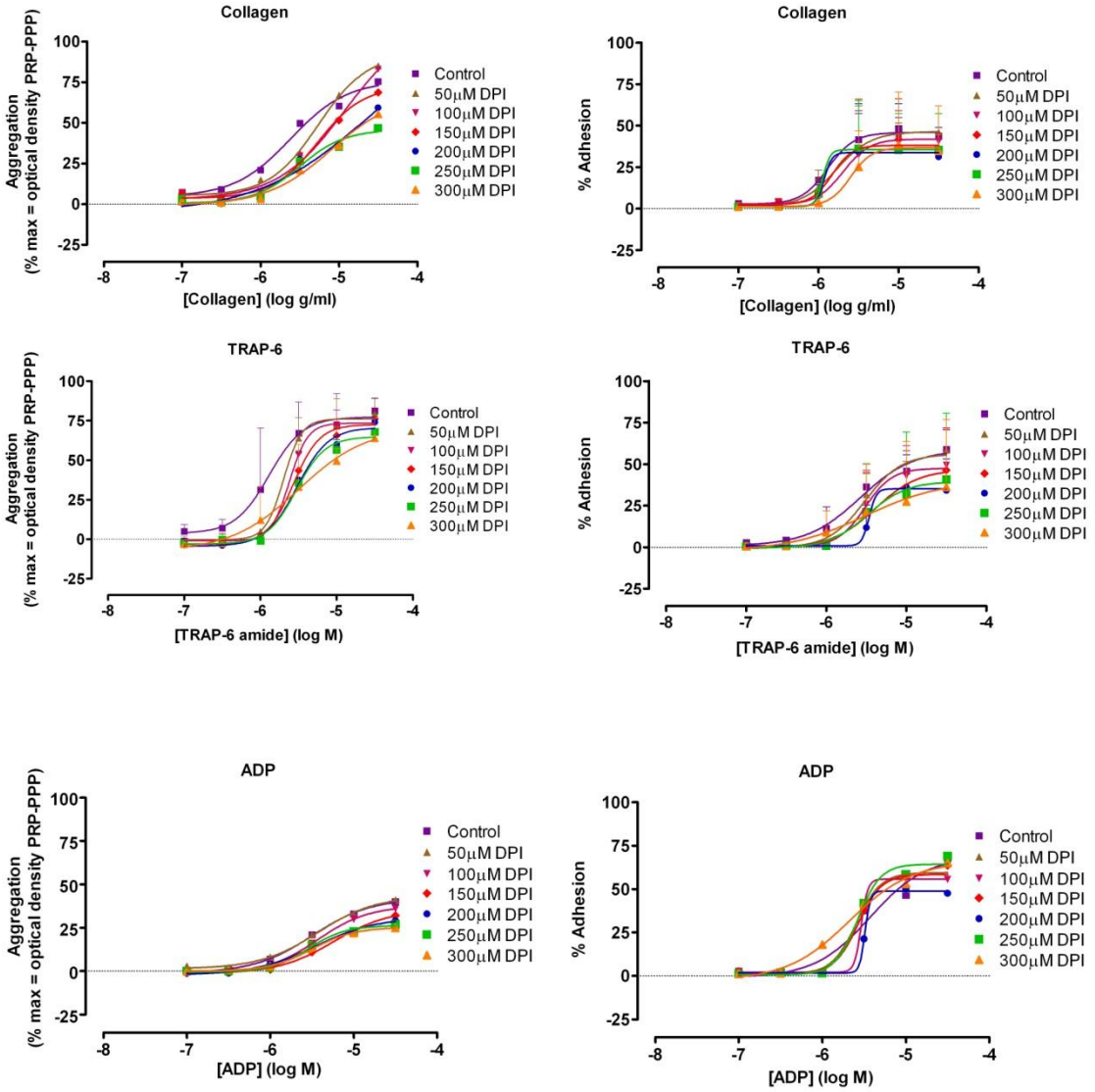


Figure 4.20: *Concentration dependent effects of DPI on platelet aggregation and adhesion stimulated by collagen, TRAP-6 amide and ADP*. PRP was incubated with 50-300μM DPI before agonist-stimulated platelet aggregation was measured in 96-well plates. Each data point represents mean ± S.E.M. (n=1-3).

4.3.9 Effects of Combination of CDC and DPI on Platelet Activation Stimulated by Various Agonists

Combination of CDC, a 12-LOX inhibitor and DPI, used as a NADPH oxidase inhibitor inhibited platelet aggregation in response to ADP and collagen, whilst aggregations to other agonists were not affected (Figure 4.21). For instance, 10 and 30μM ADP induced platelet aggregations of 31±5 and 47±8% in the presence of CDC alone, but these were reduced to 18±3% and 23±3%, respectively, when CDC was combined with DPI. Upon stimulation by 10 and 30μg/ml collagen, platelets incubated with CDC alone demonstrated aggregation to 54±12% and 81±35 that were decreased following combined treatment to 41±10% and 53±6%. For platelet adhesion, combination of CDC and DPI significantly decreased ristocetin and TRAP-6 -induced adhesion as well as that to ADP and collagen, as shown in Figure 4.22. For instance, in the presence of CDC alone, adhesions of platelet stimulated by 1.0 and 2.0mg/ml ristocetin were 19±11% and 22±12% which were reduced to 0% and 19±9%. The inhibition of ristocetin-induced platelet adhesion by DPI is in accord with previous data in this study that found DPI inhibited platelet adhesion but not platelet aggregation. Similar findings were also demonstrated in TRAP-6–induced platelet activation where inhibition was more against adhesion than aggregation.

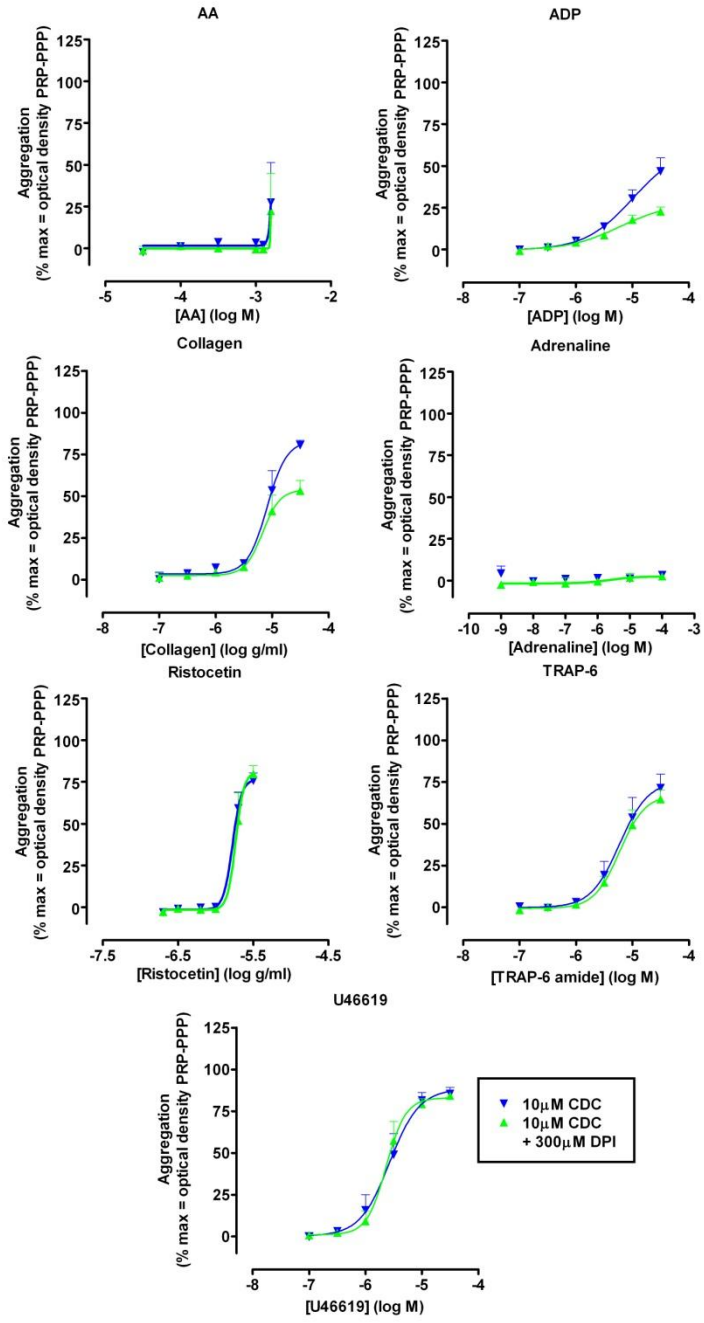


Figure 4.21: *Effects of combination of CDC, a 12-LOX inhibitor and DPI, a NADPH oxidase inhibitor on platelet aggregation and adhesion stimulated by various agonists*. PRP was incubated with 10μM CDC with or without the combination of 300μM DPI before agonist-stimulated platelet aggregation was measured in 96-well plates. Each data point represents mean ± S.E.M. (n=4).

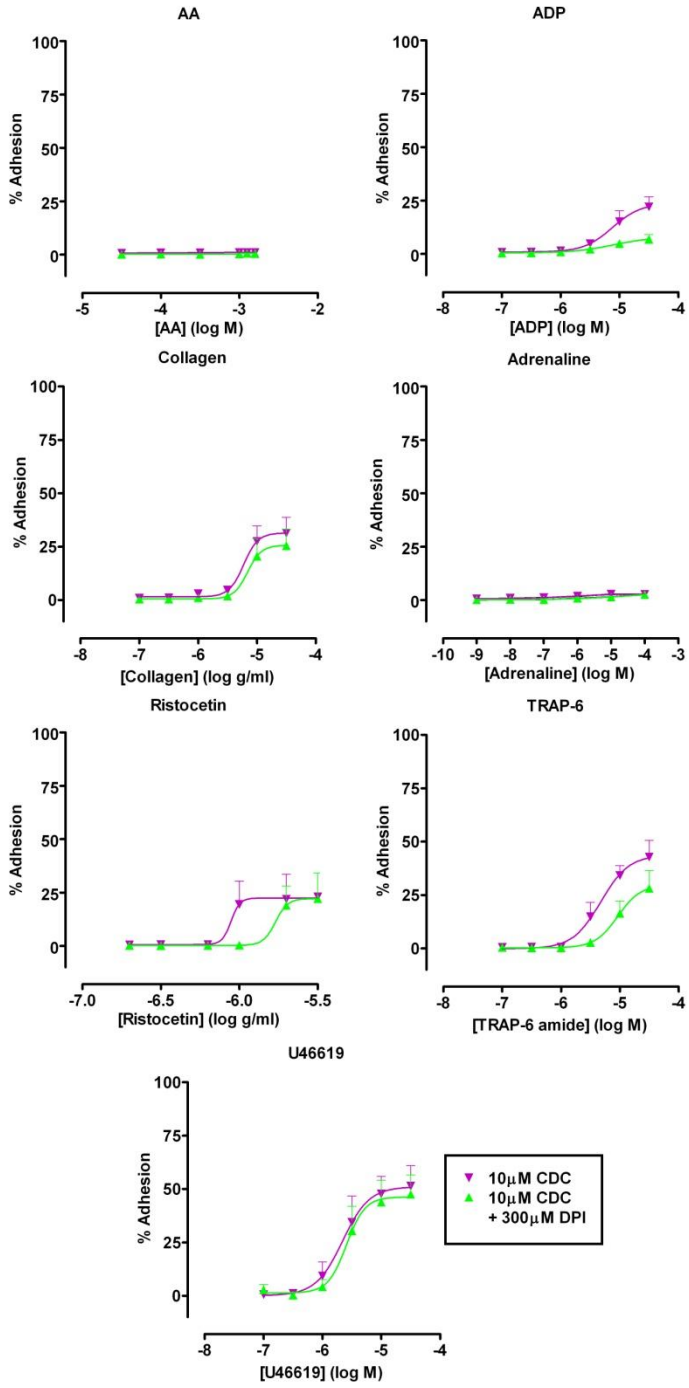


Figure 4.22: *Effects of combination of CDC and DPI on platelet adhesion stimulated by various platelet agonists*. Each data represents mean ± S.E.M. (n=4).

4.3.10 Effects of Apocynin, a NADPH Oxidase Inhibitor, on Platelet Activation by Various Agonists

Platelet aggregations in response to ADP, collagen and TRAP-6 were decreased by apocynin whilst those to other agonists' stimulation did not show any changes (Figure 4.23). For instance, aggregations to 3µM ADP, 23±15%, and 3µM collagen, 22±11%, were decreased to 8±5% and 10±1%, respectively, by apocynin. Similar observations were seen for aggregation induced by adrenaline at 1, 10 and 100µM which were decreased from 19±17%, 17±15% and 21±17% to 1±3%, 9±11% and 11±14%, respectively. Platelet adhesion was much more affected by apocynin compared to aggregation, as shown in Figure 4.24, with adhesion to other agonists, ristocetin and TRAP-6, being affected in addition to those to ADP, collagen and adrenaline. Although inhibition of platelet adhesion by apocynin was not great, the pattern of inhibition was similar to that for DPI, which also reduced adhesions of platelets challenged by the same agonists.

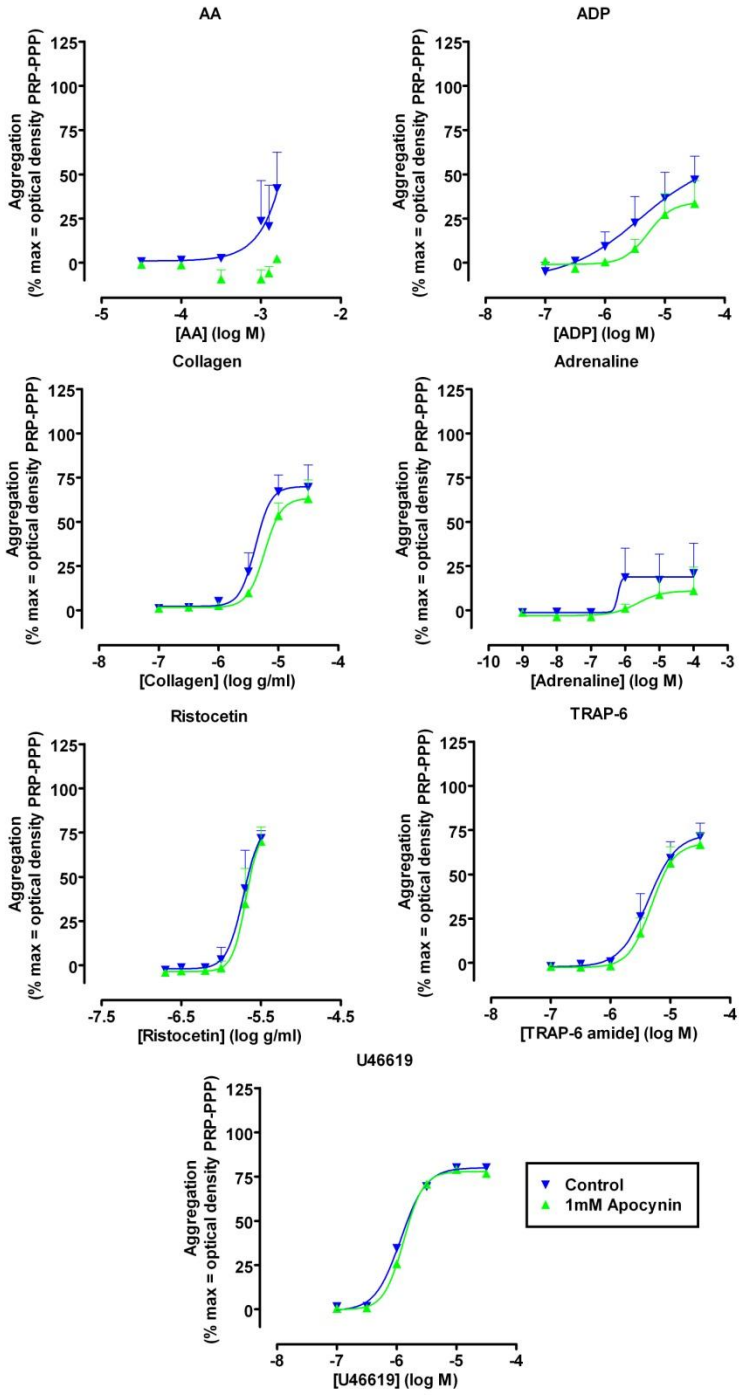


Figure 4.23: *Effects of apocynin on platelet aggregation and adhesion stimulated by various agonists*. PRP was incubated with 1mM apocynin before agonist-stimulated platelet aggregation was measured in 96-well plates. Each data point represents mean ± S.E.M. (n=4).

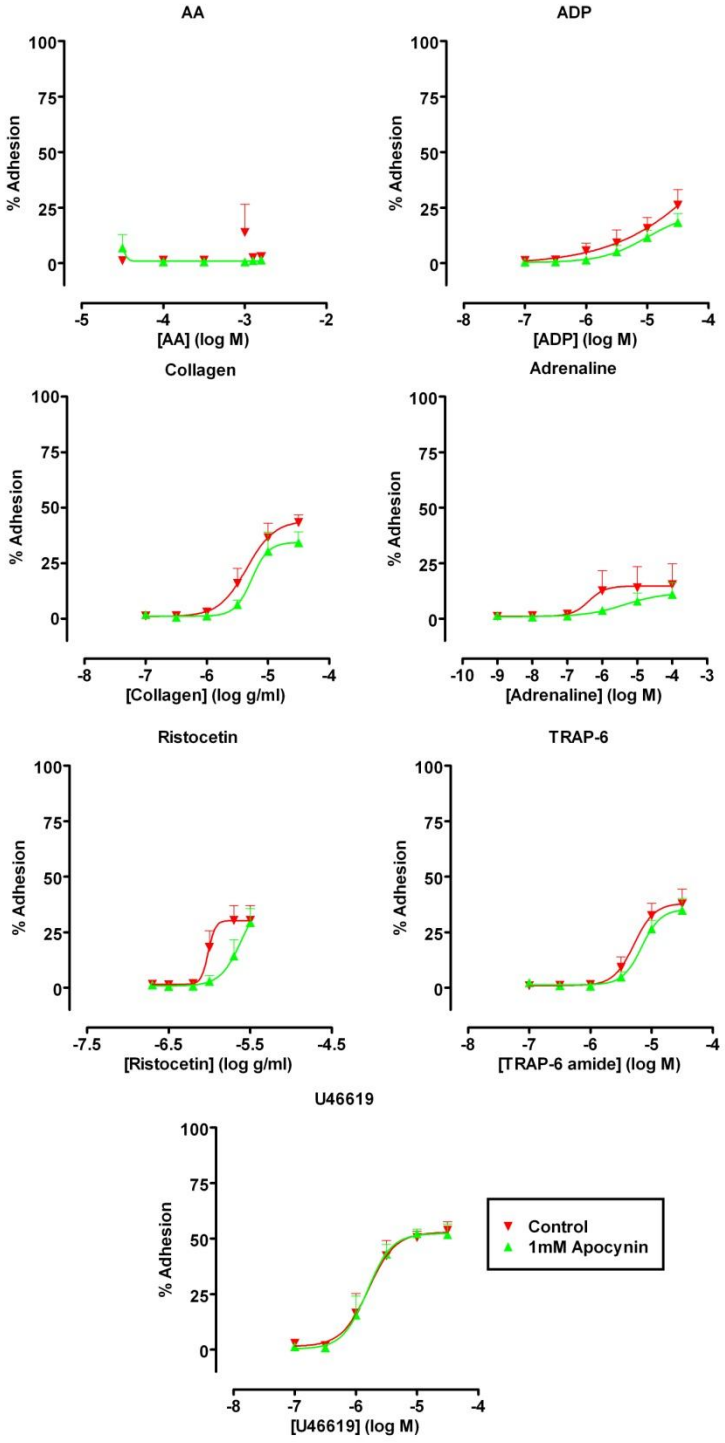


Figure 4.24: *Effects of apocynin on platelet adhesion stimulated by various platelet agonists*. Each data point represents mean ± S.E.M. (n=4).

4.4 Discussion

The results presented here demonstrate that the platelet 12-LOX pathway is involved in platelet responses to AA, adrenaline, ADP, collagen and ristocetin, although the effects on aggregation and adhesion are generally weak. However, the variability of the effects of the 12-LOX inhibitors, CDC and baicalein, suggest that the action is different for different agonists. The data also suggests that when platelets are activated by low concentrations of collagen or adrenaline 12-LOX products are more involved in the primary platelet activation than following stronger platelet activation, such as by combination of collagen and adrenaline.

Several studies have investigated the activation of platelet 12-lipoxygenase (p12-LOX) following platelet stimulation by agonists. 12(S)-HPETE synthesis is activated in washed human platelets stimulated by collagen and collagen reactive peptides (CRP) (Coffey *et al.*, 2004b). However, this study also showed that ADP and U46619 alone did not activate p12-LOX pathway whilst a high concentration of thrombin (>0.2 U/ml) is required to activate 12(S)-HPETE synthesis. Further, *src*-tyrosine kinases, PI3 kinase, Ca^{2+} mobilization and p12-LOX translocation is essential for p12-LOX activation by the GPVI collagen receptor, which, however, is down regulated by PKC and PECAM-1 (Coffey *et al.*, 2004b). As 12(S)-HPETE is produced in dose- and time-dependent manner following activation by CRP, this suggested that GPVI alone is sufficient for the activation of p12-LOX. P12-LOX is unable to

directly oxidise AA that is esterified to membrane phospholipids. Therefore, phospholipase A_2 (PLA_2) is required to provide the supply of AA for p12-LOX product synthesis. Several PLA_2 isoforms are expressed in platelets such as $cPLA_2$, calcium-independent ($iPLA_2$) and secretory phospholipase ($sPLA_2$). However, only c- and $sPLA_2$ are responsible for the AA liberation leading to 12(S)-HPETE generation following GPVI collagen receptor activation in washed human platelet (Coffey *et al.*, 2004a).

The role of the LOX pathway in platelet function is unclear and continually debated. Platelet aggregation requires Ca^{2+} mobilization; however, indomethacin attenuated ADP-induced platelet aggregation without affecting Ca^{2+} signal generation suggesting that the COX pathway is not the prerequisite (Hallam *et al.*, 1985). Moreover, previous investigations have shown that both LOX inhibitors, nordihydroguaiaretic acid (NDGA) and BW-755C suppressed ADP-induced platelet aggregation and Ca^{2+} signals dose dependently (Borin *et al.*, 1989). Thus, this study suggested that LOX pathway may have an important role in ADP-induced Ca^{2+} mobilisation and platelet aggregation. In contrast, more recent reports have concluded that products from the LOX pathway are not responsible for the ADP-induced Ca^{2+} mobilisation and platelet aggregation (Vindlacheruvu *et al.*, 1991). This investigation involved the use of more specifics LOX inhibitor than NDGA, BW A4C and MK 866, on basal Ca^{2+} signal generation and platelet aggregation stimulated by ADP, which was found not affected by these two compounds. On the contrary, treatment with aspirin or indomethacin reduced Ca^{2+} signal generation. Overall, therefore, the involvement of

p12-LOX pathway on platelet aggregation and Ca^{2+} mobilisation upon ADP stimulation is still unclear (Vindlacheruvu *et al.*, 1991).

In addition, the hypothesis that p12-LOX pathway is involved in platelet activation is contradicted by the findings by Johnson and colleagues who reported an anti-aggregatory effects of the LOX pathway (Johnson *et al.*, 1998). This study was performed in mice with disrupted platelet LOX gene (P-12LO$^{-/-}$) that had hypersensitivity to ADP-induced aggregation and an increased mortality in ADP-induced models of thrombosis (Johnson *et al.*, 1998). Even though this finding provides further evidence of the suppressive effects of LOX products in platelets, this conclusion has to be balanced by consideration of the species and agonists used in the study. Moreover, AA liberation in thrombin-stimulated human platelets results in the synthesis of both COX and LOX products which were dose-dependently reduced by BW 755C (Smith *et al.*, 1985), as BW-755C is a combined LOX and COX inhibitor, suggesting that products from both pathways are responsible for the effects seen. This is in agreement with a previous study which reported that TXB_2 and 12(S)-HETE are elevated after thrombin-induced aggregation of washed platelet (Hamberg *et al.*, 1974). Moreover, another investigation reported an inhibition of thrombin and U46619-induced aggregation in human washed platelets by 5,8,11-eicosatriynoic acid (ETI), baicalein and phenidone (Nyby *et al.*, 1996). Nyby *et al.* (1996) also suggested that LOX inhibitors may play a role in the regulation of cyclic AMP metabolism.

Overall, there is still lack of agreement as to whether LOX pathway is involved in the regulation of platelet function. However, a recent study has reported the pro-atherogenic co-effects of TP receptor signalling and 12/15 LOX gene disruption *in vivo* suggesting the importance of both pathways as therapeutic targets for the treatment of atherosclerosis (Tang *et al.*, 2008). In addition, 12(S)-HETE is suggested to play a role as a modulator of the expression of P-selectin (CD-62), following observation of thrombin's ability to increase 12(S)-HETE levels and P-selectin expression while not affecting TXA_2 (Ozeki *et al.*, 1998). Interestingly, the LOX inhibitors, quercetin and NDGA inhibited P-selectin expression whereas the COX inhibitors, indomethacin and aspirin, did not. Thus, the addition of 12(S)-HPETE and 12(S)-HETE were found to enhance P-selectin expression, suggesting both 12-LOX metabolites are involved in platelet activation (Ozeki *et al.*, 1998).

LOX inhibitors are important to the elucidation of the biological significance of LOX products. Catecholic antioxidants, nordihydroguaiaretic acid, NDGA or 5,8,11,14 eicosatetraynoic acid, ETYA are known as non-specific LOX inhibitors; both were widely used in early research of the LOX pathway, regardless of the type of LOX and its isoforms. Baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one) is a phenolic flavonoid compound that is found in abundance in Huang Qin (*Scutellaria baicalensis* Georgi), a Chinese medicinal plant. This compound has been shown to exert wide biological effects including anticancer, antiviral, antithrombotic and anti-inflammatory (Lee *et al.*, 2011),. In most all these cases the effects of baicalein are thought to be explained by selective inhibition of p12-LOX (Deschamps *et al.*, 2006). However, Deshamps and colleagues (2006)

have showed that baicalein is unselective between platelet 12-LOX and reticulocyte 15-LOX *in vitro*.

Previous significant research has been done to study the formation of hydroxyl radical by baicalein using Electron Spin Resonance (ESR) Spectophotometry in human platelet suspensions (Chou *et al.*, 2007). This study found that baicalein induced hydroxyl radical formation in resting human platelets which was enhanced by addition of low but not high concentrations of AA. It has also been shown that the hydroxyl radical formation induced by baicalein is due to the p12-LOX pathway and that the liberation of AA is essential for the activation of p12-LOX. It is noteworthy that in this paper, a high concentration of baicalein, 300µM was used to compensate for the competitive nature of baicalein. Interestingly, this study suggested p12-LOX dependent roles of baicalein as antioxidant or as a pro-oxidant in human platelet suspension (Chou *et al.*, 2007). Following this study, another investigation has been performed using B16F10 melanoma cells and baicalein has been shown to produce hydroxyl radical in these cells (Chou *et al.*, 2009). More importantly, the viability of these cells was reduced by baicalein along with an increase in the formation of hydroxyl radical without alteration in 12-LOX protein expression. A growing number of investigations of the importance of 12-LOX in cardiovascular problems have involved studies of baicalein, for example in *Chlamydia pneumoniae* activated platelets (Kälvegren *et al.*, 2007). This study has used various pharmacological agents and found that 12-LOX, purinergic receptors and PAF but not COX, are important in harmful *C. pneumoniae* induced platelet activation. In contrast, baicalein and another 12-LOX

inhibitor, cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) were found to have no effects on collagen-stimulated platelets. Baicalein was suggested to be useful as a novel treatment for acute ischemic stroke (Lapchak *et al.*, 2007) and to improve myocardial contractility in LPS-induced sepsis in vivo (Lee *et al.*, 2011). Recently, a cathechin from tea leaves, (-)-epigallocatechin gallate, was found to be a very potent and highly selective inhibitor of human p12-LOX (Yamamoto *et al.*, 2005).

12-LOX metabolism of arachidonic acid metabolism in platelets results in the formation of 12-(S)-hydroxyperoxy-eicosatetraenoic acid [12-(S)-HPETE]. The latter is further reduced to 12-hydroxy-eicosatetranoic acid (12(S)-HETE) by glutathione peroxidase, explaining the unstable and short life-span of 12(S)-HPETE. Results presented here show that 12(S)-HPETE may inhibit platelet function following activation by AA and decrease ristocetin-stimulated platelet adhesion, whilst activation by other agonists is not affected. An earlier study by Siegel *et al.* (1979) suggested that 12(S)-HPETE is involved in the regulation of platelet AA metabolism. By using platelet homogenates, this study demonstrated that 12(S)-HPETE increased the 12-LOX activity but not 12(S)-HETE, and decreased the activity of platelet COX-1 and TXB_2 formation (Siegel *et al.*, 1979). Unfortunately, studies on physiological effect of 12(S)-HPETE on platelets are limited and controversial. However, 12(S)-HPETE has been reported to inhibit platelet aggregation stimulated by AA and collagen in PRP using traditional aggregometry (Siegel *et al.*, 1979). The inhibitory effect of 12(S)-HPETE has also been extensively investigated by Aharony and his colleagues using washed platelets (Aharony *et al.*, 1982). Inhibitory effects of 12(S)-HPETE on platelet

aggregation and platelet secretion in response to AA, U46619 and collagen were found. In contrast, thrombin-induced platelet aggregation was not affected but platelet secretion was reduced after treatment with 12(S)-HPETE. Further, this study showed that platelet secretion is reduced in untreated and aspirin-treated volunteers suggesting that inhibition by 12(S)-HPETE of platelet secretion is independent of platelet COX (Aharony *et al.*, 1982). These findings were supported by further research that found that aggregation in response to U46619 and thrombin of platelets in PRP from aspirin-treated donors were inhibited (Brune *et al.*, 1991).

Even though previous studies have mainly reported inhibitory effects of 12(S)-HPETE on platelet aggregation, there is an evidence of 12(S)-HPETE stimulating platelet function for instance in the presence of low non-aggregating concentrations of AA (Calzada *et al.*, 1997). In this study, concentration-dependent platelet aggregation was demonstrated to 12(S)-HPETE, 0.5-2μM, with the effect being dependent upon COX activity but not via a receptor-mediated mechanism. Therefore, is has been concluded that 12(S)-HPETE is pro-aggregatory at low concentrations close to those seen physiologically, although inhibitory effects are observed at higher concentrations of 12(S)-HPETE (Calzada *et al.*, 1997). A further study by Calzada *et al.* (2001) has shown that 12(S)-HPETE increases platelet aggregation and TXA_2 formation to sub-threshold concentrations of collagen in washed platelet (Calzada *et al.*, 2001). Moreover, this study found that 12(S)-HPETE is involved in the liberation of nonesterified AA by translocation and phosphorylation of $cPLA_2$. This study suggested that 12(S)-HPETE is responsible for the regulation of endogenous AA and

TXA_2 formation and so influences platelet function. However, this conclusion is only applicable in the nanomolar range of 12(S)-HPETE concentrations used in this studies; previous studies by Siegel *et al.* (1979), Aharony *et al.* (1982) and Brune *et al.* (1991) used 12(S)-HPETE at micromolar concentrations. Similarly, the results presented here suggest the importance of the pro-platelet effects of 12(S)-HETE in the presence of low concentration of platelet agonists, notably adrenaline and collagen.

The biological significance of 12(S)-HETE on platelets is still unknown although an inhibitory effects of 12(S)-HETE on platelet aggregation has been suggested by many studies (Takenaga *et al.*, 1986; Sekiya *et al.*, 1990; Croset *et al.*, 1988; Fonlupt *et al.*, 1991). Meanwhile, previous studies have also been conducted to compare the LOX metabolites formed from AA and eicosapentanoic acid (EPA) on human platelet function, because EPA has been reported to have an anti-platelet affects by several investigators (Takenaga *et al.*, 1986). For these studies LOX metabolites, 12(S)-HPETE and 12(S)-HETE derived from AA whilst 12(S)-HPEPE and 12(S)-HEPE produced from EPA were isolated from human platelet lysates. Takenaga *et al.* (1986) found that addition of 12(S)-HPETE and 12(S)-HPEPE reduced both platelet aggregation and 5-HT release induced by AA and collagen in concentration-dependent manners. This study also reported that both 12(S)-HETE and 12(S)-HEPE demonstrated weaker inhibitory effects on platelet function.

Another study proposed an inhibitory effect of 12(S)-HETE on platelet aggregation, based on enhanced collagen-induced aggregation and AA liberation in bovine platelets in the

presence of the 12(S)-HETE inhibitor, 15-HETE (Sekiya *et al.*, 1990). Moreover, this study reported exogenous addition of 12(S)-HETE attenuated collagen-induced aggregation and liberation of AA. Therefore, Sekiya and colleagues inferred that 12(S)-HETE is involved in a negative feedback loop upon platelet function. This is possible as during the increase of AA liberation and TXA_2 production that follows stimulation of platelet aggregation, 12(S)-HETE also accumulates and so could interfere aggregation by inhibiting further AA liberation from platelet membrane. This is also supported by another study that found U46619-induced platelet aggregation is inhibited by 12(S)-HETE (Croset *et al.*, 1988). Subsequent studies showed that 12(S)-HETE inhibited the binding of radio-labelled thromboxane mimetic, U44069 at the receptor sites suggesting the inhibitory effects of 12(S)-HETE might be because of explained by blocking of PGH_2/TXA_2 receptor sites (Fonlupt *et al.*, 1991). The potentiating effects of 12(S)-HETE on collagen and U46619 are contradicted by another study using different platelet agonist. It was demonstrated that 12(S)-HETE enhanced bovine and human platelet aggregation induced by thrombin with PGE_1-induced cGMP elevation being abolished by 12(S)-HETE (Sekiya *et al.*, 1991). However, another evidence of proaggregatory effects of platelet 12(S)-HETE was published after confirming a regulatory role of 12(S)-HETE.

A newly specific 12(S)-HETE synthesis inhibitor, *(S)*-(+)-6-[3-(1-*o*-tolylimidazol-2-yl)sulfinylpropoxy]-3,4-dihydro-2(H)-quinolinone (OPC-29030) has been found to reduce *ex vivo* platelet aggregation and 12(S)-HETE formation following stimulation of platelets with ADP and U46619 (Katoh *et al.*, 1998). In addition, activation of platelet gpIIb-IIIa upon

stimulation by ADP, U46619 and thrombin was shown to be reduced by baicalein and OPC-29030 in the same study. The expression of platelet P-selectin was also suggested to be involved in the activation of platelets by 12(S)-HETE following the finding that non-specific 12-LOX inhibitors, quercetin and nordihydroguiaiaretic acid but not aspirin decreased P-selectin expression following stimulation with thrombin (Ozeki *et al.*, 1998). The use of OPC-29030 has been further explored with another finding suggesting that the translocation of 12-LOX from cytosol to the membrane is required for the generation of 12(S)-HETE (Ozeki *et al.*, 1999).

The significance of 12(S)-HETE on platelet function has been indirectly investigated by many research groups in various conditions. Increase levels of 12(S)-HETE have been suggested in hypertension and to acts as a vasoconstrictor in small arteries. Although thrombin stimulation did not increase platelet 12(S)-HETE generation in samples from normal and hypertensive patients, the basal platelet 12(S)-HETE levels, urinary 12(S)-HETE and platelet 12-LOX expression are higher in patients than normal (Gonzalez-Nunez *et al.*, 2001). The activation and recruitment of thrombin-induced platelets is inhibited by unstimulated neutrophils. However, these effects are reduced in the presence of ETYA suggesting 12-LOX products are important in thrombin-stimulated platelet thrombus formation (Valles *et al.*, 1993). Previous findings also showed that 12(S)-HETE is involved in tumor-induced platelet aggregation with activation of gpIIb-IIIa (Steinert *et al.*, 1993), and that low 12(S)-HETE levels in neonates is associated with bleeding tendencies (Fretland *et al.*, 1989).

In the studies presented here evidence points to the fact that when the platelet COX pathway is inhibited, platelet shifts to become more dependent on the 12-LOX pathway, although this appears to apply only when platelets are activated by AA. The relationship between COX and LOX pathway in platelets is highly debated and there is lack of concrete evidence. Earlier *in vitro* studies by McDonald-Gibson and colleagues demonstrated attenuation of 12(S)-HETE production by the same concentration of aspirin that is required to inhibit platelet aggregation but not by the aspirin concentration that inhibits TXA_2 production in human washed platelets (McDonald-Gibson *et al.*, 1984). This study suggested a possible role for the LOX pathway in aggregation of washed platelets in buffer, independent of the platelet COX pathway. The role of the LOX pathway was further explored by Eynard *et al.* (1986), who looked into the effects of aspirin on 12(S)-HETE formation in human and *in vitro*. This study reported that following administration of low (20mg) and high (500mg) doses of aspirin to healthy volunteers, platelet aggregation, TXA_2 and 12(S)-HETE levels were significantly decreased (Eynard *et al.*, 1986). *In vitro* data in this study also suggested that plasma is a prerequisite for the aspirin effect on LOX products as seen by the inhibition of formation of 12(S)-HETE in PRP, but not in washed platelets. This study is supported by another *in vitro* investigation that found inhibitory effects of aspirin, indomethacin and BW 755C but not sodium salicylate on 12(S)-HETE synthesis on collagen-stimulated PRP (Tremoli *et al.*, 1986). Therefore, it can be concluded that aspirin not only inhibited COX pathway but also caused reversible effects on the LOX

pathway, both *in vitro* and *ex vivo*, with 12(S)-HETE synthesis being constantly inhibited over a 24 hour period following administration of aspirin (Maderna *et al.*, 1988).

All of these studies, however, did not investigate the relationship of COX and LOX in terms of platelet function, which is important in determining the relative biological effects of the two pathways in platelets. A previous earlier study investigated the effects of the LOX inhibitors, ETYA and NDGA, on platelet function measured as aggregation and adhesion of PRP (Gimeno *et al.*, 1983). This found that the aggregation response to collagen and ADP of PRP from aspirin-treated patients was reduced after pre-incubation with ETYA or NDGA and that the same was true for adhesion in response to collagen. As a result, this study concluded that LOX pathway and its product are essential to the mechanism of platelet function, particularly platelet aggregation and adhesion (Gimeno *et al.*, 1983). Another study used human washed platelet suspensions to determine the effects of aspirin and salicylate treatment on platelet aggregation and adhesion along with TXA_2 and 12(S)-HETE formation (Buchanan *et al.*, 1986). This study found that aspirin decreased platelet aggregation associated with TXA_2 production but increased adhesion associated with an increase in 12(S)-HETE formation. In contrast, following salicylate treatment, platelet aggregation was increased as TXA_2 production was higher than control; however 12(S)-HETE formation was decreased as expected resulting in decreased platelet adhesion. These observations may be explained by the shift of AA metabolism to LOX pathway as a result of COX pathway inhibition suggesting endogenous 12(S)-HETE facilitates platelet adhesion which can be reduced by treatment with salicylate.

It is interesting to note that AA can also be metabolized by cytochrome P450 in which its products are derived from the NADPHdependent P450 catalyzed insertion of a single atom of oxygen into the AA molecules (Figure 4.25) (Capdevila *et al.*, 2000). In contrast, the reactions catalyzed by COX and LOX are initiated by regioselective hydrogen atom abstraction from a *bis*-allylic methylene carbon, followed by regio- and enantioselective coupling of the resulting carbon radical to ground-state molecular oxygen (Smith *et al.*, 1991). Both COX and LOX metabolism are also NADPH-independent, thus they are dioxygenases that catalyze substrate carbon activation whilst P450 are typical monoxygenases (Smith *et al.*, 1991). P450-derived products are epoxy- and hydroxy-metabolites of AA (EETs and HETEs) that involve in the conversion of cholesterol, steroids, bile acids, vitamins, and xenobiotics and also generate biologically relevant amounts of ROS (Capdevila *et al.*, 2002).

EETs cause membrane hyperpolarization in endothelial and smooth muscle cells via regulation of calcium-permeable plasma membrane channels. In addition to regulating calcium concentration and membrane potential, EETs also induce tyrosine kinase, p42/44, and p38 activity in EC (Hoebel *et al.*, 1998). In contrast to EETs, 20-HETE inhibits Ca^{2+}-dependent K^{+} channels, inducing depolarization, and increases intracellular calcium concentration through voltage-sensitive calcium channels (Kroetz *et al.*, 2002). Interestingly, overexpression of epoxygenases and exogenous addition of EETs has been demonstrated to increase eNOS expression and its activity, consequentially raising NO biosynthesis in endothelial cells *in vitro* (Wang *et al.*, 2003). Furthermore, 11,12-EET has

been reported to increase NOS activity in human platelets *in vitro* (Zhang *et al.*, 2008). Therefore, overexpression of epoxygenases may promotes anti-atherosclerotic effects that are correlated with elevated EET levels and associated with improvement of endothelial function (Xu *et al.*, 2011).

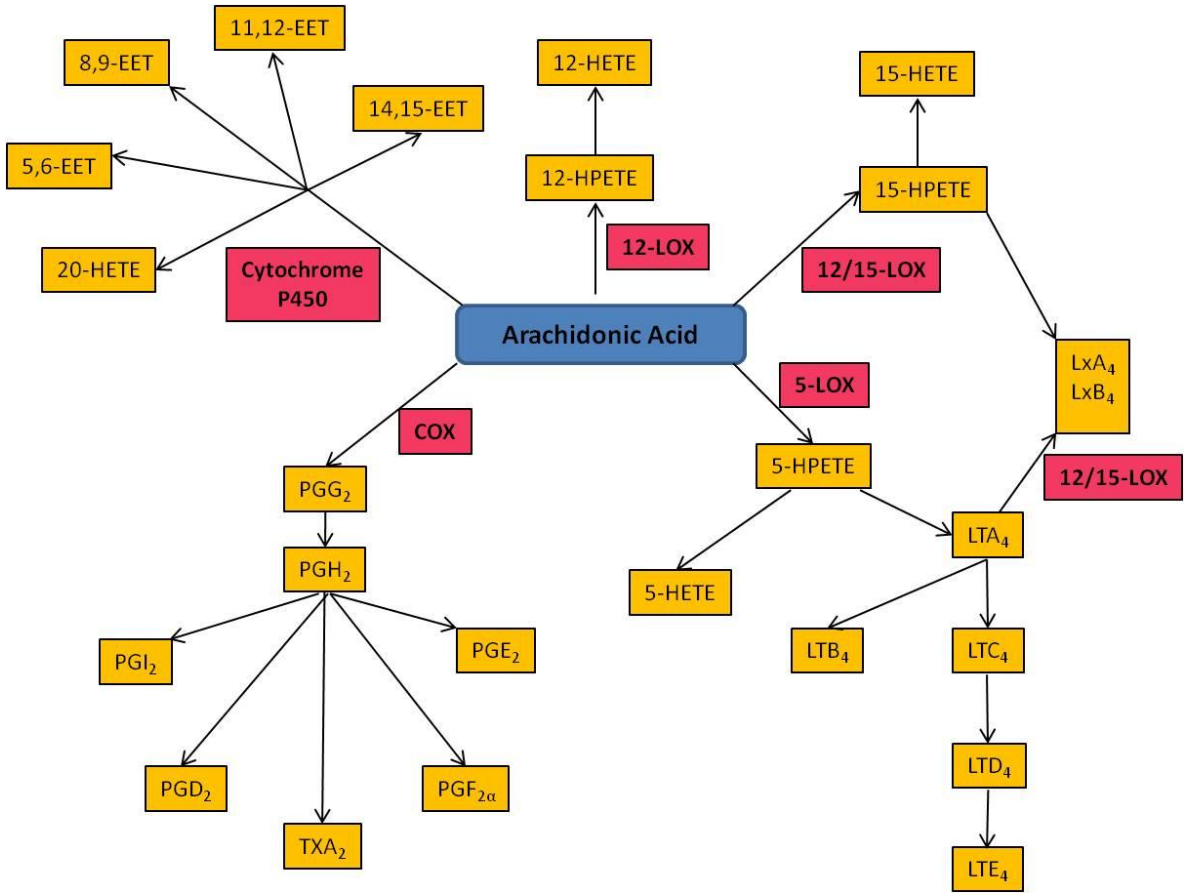


Figure 4.25: *The arachidonic acid cascade.* This schematic diagram depicts arachidonic acid conversion products. Names of primary enzymes are in pink box under the appropriate arrow. PG, prostaglandin; LT, leukotriene; H(P)ETE, hydro(pero)xyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; Lx, lipoxin; COX, cyclooxygenase, LOX, lipoxygenase; Cytochrome *P*450, cytochrome *P*450 oxygenase. Adapted from Bogatcheva *et al.(*2005).

In order to determine the possibility of NADPH oxidase involvement in platelet aggregation and adhesion, studies here examined the effects of DPI and apocynin upon platelet activation. The results suggested that blocking NADPH oxidase reduces the ability of platelets to aggregate but that this effect is limited to specific agonist pathways as the inhibition is clearly observed in ADP, collagen, adrenaline and TRAP-6 but not in AA, U46619 and ristocetin. In contrast, platelet adhesion was found to be more sensitive to treatment with NADPH oxidase inhibitors, with ristocetin-induced adhesion being decreased.

It is strongly suggested that activated platelets produce reactive oxygen species (ROS) and that these are important for the regulation of platelet function. For instance, platelet activation induced by thrombin, TRAP-6, U46619 and convulxin but not ADP showed a similar elevations in ROS production (Begonja *et al.*, 2005). ROS is synthesised in platelet by various cytosolic enzymes, such as xanthine oxidase, monoamine oxidase, LOX, the endothelial isoform of nitric oxide (eNOS) and membrane electron transfer system (Del Principe *et al.*, 2009).

It was reported that superoxide production by stimulated platelets increases platelet aggregation and adhesion (Salvemini *et al.*, 1989), adding to our understanding of the pro-aggregatory effects of ROS. A previous study has shown that platelet-derived ROS is generated by the activity of intracellular platelet nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) (Seno *et al.*, 2001).

NADPH oxidase produces ROS via a one electron reduction of molecular oxygen. NADPH oxidase is a multicomponent enzyme, consisting of the membrane bound cytochrome b_{558} (composed of the two subunits gp91-phox and p22-phox) and the cytosolic proteins, p47-phox, p67-phox, p40-phox, and small GTP-binding protein Rac. The common NADPH oxidase inhibitors used in research are DPI and apocynin. Apocynin is also known as acetovanillone or 1-(4-hydroxy-3-methoxyphenyl)ethanone and acts to block oxidase assembly. Seno *et al.* (2001) demonstrated ROS production in washed platelets upon stimulation by calcium ionophore, which is known to be a potent activator of phagocytic NADH/NADPH oxidase. In this study, it was shown that platelet ROS production is generated largely via NADPH oxidase activity rather than xanthine oxidase activity, supported by the expression of p22-phox and p67-phox proteins in platelets. NADPH oxidase activation involves the phosphorylation and translocation of the cytosolic components to the membrane-bound cytochrome b_{558} where the catalytically functional oxidase is assembled. In platelets, inhibition of phosphoinositide 3-kinase (PI3-kinase) by NO leads to reduction in functional NADPH oxidase and thus contributes to reduction in platelet activation (Clutton *et al.*, 2004). Therefore, it can be concluded that NADPH oxidase is an important enzyme responsible for platelet ROS generation and plays an important role in the regulation of platelet function.

Angiotensin II has been shown to increase superoxide anion production by platelet through PKC-dependent NADPH oxidase activation (Plumb *et al.*, 2005). Although the enzymes underlying ROS generation in platelets are not well defined, AA metabolism has been shown to produce ROS in collagen-stimulated platelets, and this effect is dependent upon the AA concentration. In addition, both COX and LOX pathways are able to produce ROS, as inhibitors of either pathway caused decreases in ROS production (Seno *et al.*, 2001). Therefore, a dual major pathway in platelet has been suggested to generate ROS, which is the NADPH oxidase pathway, and AA metabolism consisting of COX and LOX pathways. A previous study by Plumb *et al.* (2005) has confirmed the expression of gp91-phox subunit in platelets taken from patients with essential hypertension. Additionally, superoxide anion generation is in complete absence following stimulation by collagen, thrombin and AA of platelets taken from patients with gp91-phox deficiency (Pignatelli *et al.*, 2004).

As the importance of LOX metabolites in regulating platelet function is still controversial, the relationship of between ROS generation and the LOX and NADPH oxidase pathways remain elusive. There is evidence showing that 12(S)-HETE play a role in the activation of NADPH oxidase. It has been demonstrated that 12(S)-HETE stimulates ROS generation, platelet fragmentation and platelet microparticle formation in normal human and mouse platelets, however platelets from gp91phox-/-, Rac 2-/- and 12LO-/- mice failed to demonstrate these effects (Nardi *et al.*, 2004). The formation of platelet particles is suggested to be dependent upon the NADPH oxidase pathway as exogenous 12(S)-HETE

induces particle formation in 12LO-/- mouse platelets. This is supported by another finding by Nardi *et al.* (2007) that inhibition of platelet particle formation by dexamethasone following induction by either anti-GPIIIa49-66 antibody, calcium ionophore or phorbol myristate acetate is due to its blocking of the translocation of PLA_2, 12-LOX and p67-phox from the cytosol to platelet membranes (Nardi *et al.*, 2007). Based on these findings, it can be inferred that platelet PLA_2, 12-LOX and NADPH oxidase modulate platelet ROS generation that is involved in platelet particle formation. Data reported here shows that in the presence of LOX inhibition, NADPH oxidase inhibition can still inhibit platelet function. It therefore seems that the role of platelet NADPH oxidase in regulating platelets activity is at least partly independent of platelet 12-LOX.

The ROS production by platelet NADPH oxidase has been shown to regulate intergrin αIIbβ3 activation but not platelet granule secretion and shape change (Begonja *et al.*, 2005). In this study, the possible source and type of intracellular ROS in platelet was examined using various inhibitors and superoxide scavengers, including DPI and apocynin. Begonja *et al.* (2005) demonstrated that thrombin-stimulated platelet ROS production rises from the NADPH oxidase pathway which was inhibited by DPI or apocynin. Interestingly, this property is shared by aspirin, thus supporting a partial involvement of AA metabolism in ROS generation. There is mounting evidence of functional platelet NADPH oxidase and ROS generation being involved in the regulation of platelet function. For instance, the inhibitory effects of NO are attenuated in the presence of ROS as reaction between NO and superoxide results in the forming of peroxynitrate ($ONOO^-$).

Taken together, this may explain the possible mechanism of ROS in promoting platelet aggregation; inhibition of ROS generation as a result of attenuation of NADPH oxidase activity will increase the dissaggregation effects of NO. The NO/cGMP pathway is an established platelet inhibitory mechanism and so this is an attractive hypothesis. However, in a previous study, cGMP levels and VASP phosphorylation failed to show any increase with DPI or apocynin, despite inhibition of platelet aggregation, suggesting NADPH oxidase regulates platelet function independent of NO (Begonja *et al.*, 2005).

In contrast with the study by Begonja *et al.* (2005), an earlier study by Krotz *et al.* (2002) found that superoxide anion could not be generated by thrombin-stimulated platelets but is in agreement that ADP-stimulated platelets do not produce ROS (Krotz *et al.*, 2002). Interestingly, ROS production has been shown to be very prominent in collagen-stimulated platelets. The source of these ROS has been demonstrated by the inhibition of superoxide anion production that follows incubation with either DPI, apocynin or specific inhibitor of NADPH oxidase, gp91ds-tat, which specifically targeting the interaction between gp91-phox and p47-phox. The results in the experiments in this thesis are in agreement with previous studies that have shown an inhibition of platelet aggregation as a result of inhibition of NADPH oxidase by DPI and apocynin. DPI in concentration-dependent manner has been demonstrated to inhibit thrombin-induced platelet aggregation in washed platelets, and inhibited ADP-induced aggregation in PRP (Salvemini *et al.*, 1991). Further, platelet aggregation, ROS generation and TXB_2 production upon collagen activation of platelets is attenuated by apocynin and this effect have also been

demonstrated in thrombin activated platelets (Chlopicki *et al.*, 2004). This is also parallel with the findings in this thesis that DPI reduces aggregation and adhesion when platelets are activated by ADP, collagen and TRAP-6.

Krotz *et al.* (2002) demonstrated that there is no influence of superoxide anions on platelet aggregation following collagen and ADP stimulation, however platelet recruitment after collagen stimulation is found to be decreased with DPI but increased with NO-synthase inhibitor *N*-nitro-*L*-arginine (*L*-NA). Thus, it is postulated that in collagen-stimulated platelet activation of NADPH oxidase, production of superoxide anions is essential in further platelet recruitment and ADP release but not in the primary aggregation. This is supported by another study that investigated the association of platelet recruitment and superoxide anion production. In this study, platelet recruitment was found to be directly correlated with platelet activation measured by PFA-100, which was inversely correlated with platelet superoxide formation (Pignatelli *et al.*, 2006). This study also shown that DPI, apocynin and polyphenols significantly reduced platelet recruitment and prolonged PFA CADP closure time. As there is evidence of platelet recruitment being inversely correlated to platelet NO, it can be postulated that these inhibitory effects could be due to reduction in platelet superoxide production leading to increased activity of platelet NO. Based on this idea, NADPH oxidase inhibitors and polyphenols were found to enhance the bioactivity of NO and eventually inhibited platelet function by inhibition of platelet superoxide. Platelet recruitment including adhesion is modulated by platelet gpIIb/IIIa expression, which is increased by platelet superoxide

(Begonja *et al.*, 2005). Pignatelli *et al.* (2006) demonstrated that polyphenols reduce collagen induced PAC binding to reduce platelet recruitment. Therefore, it can be inferred that down-regulation of platelet gpIIb/IIIa expression is involved in platelet inhibition by superoxide. This is in line with the findings in this thesis that DPI and apocynin decrease platelet adhesion but not aggregation in response to ristocetin.

In conclusion, this study has produced evidence that 12-LOX products may play a role in modulation of platelet functions, in which 12(S)-HPETE act as an antiplatelet and 12(S)-HETE may be responsible for a positive feedback mechanism when platelets are exposed to a primary agonist. NADPH oxidase, through different mechanisms, also plays a role in the pathways of platelet activation by specific agonists. In addition, 12-LOX and NADPH oxidase pathways have been demonstrated to be essential in events following ristocetin-induced platelet activation. Thus it can be inferred that 12-LOX and NADPH oxidase can be a new target for antiplatelet drugs to modify platelet reactivity, although further studies would be required to develop this idea.

CHAPTER FIVE:

THE EPICURE STUDY OF THE EFFECT OF DARK CHOCOLATE ON PLATELET FUNCTION

5.1 Introduction

Surprisingly, there is increasing evidence showing the influence of diet on cardiovascular disease particularly those with diets with high antioxidant content. For example, a diet containing fruit and vegetables, especially green leafy vegetables and vitamin C-rich fruits and vegetables exhibits cardioprotective effects against coronary heart disease (Joshipura *et al.*, 2001). Cocoa beverages were consumed by people in Mesoamerica for more than 500 years before they were brought to Europe in the 16th century, with more than 100 medicinal uses for cocoa and chocolate. *Theobroma cocoa* beans contain an estimated 380 identified chemicals and about 10 psychoactive compounds (Rusconi *et al.*, 2010). Dark chocolate, with more than 35% cocoa content, contains the highest concentration of polyphenols as compared to other cocoa derivatives. As total phenolic contents in diet may be associated with nutritional benefits, consumption of dark chocolate may have an impact on cardiovascular disease via a number of mechanisms. These include the protection of target molecules such as lipids from oxidative damage, suppression of inflammation and modulation of vascular homeostasis (Rein *et al.*, 2000b).

The benefits of cocoa have been suggested from the low prevalence of atherosclerotic disease, hypertension, diabetes and dyslipidemia in Kuna Indians living on an island off the Coast of Panama. An earlier study has also shown that there is decrease in blood pressure with a significant improvement in insulin sensitivity in healthy volunteers taking cocoa, suggesting protective effects on the vascular endothelium (Grassi *et al.*, 2005). In addition,

plasma polyphenol levels are increased following chocolate consumption, thus elevating the antioxidant activity of the plasma with a decrease in the concentration of plasma oxidation products (Rein *et al.*, 2000a). Consumption of cocoa beverage in overweight adult produces a significant improvement in endothelial function without alterating biomarkers of cardiac risk such as blood pressure, lipid profile, LDL oxidation and lipid hydroperoxides (Njike *et al.*, 2011). This result supports previous research findings demonstrating that flavonoid-rich dark chocolate improves endothelial function shown as an increase in plasma epicathechin concentrations in healthy adults, which is independent of plasma oxidation (Engler *et al.*, 2004). Quiñones *et al.* (2010) demonstrated the antihypertensive effects of short-term and long-term cocoa treatment in spontaneous hypertensive rats (SHR). However, recent findings concluded that the protective effect of dark chocolate on endothelial function is mediated by an increased endothelial release of nitric oxide. Interestingly, the level of oxidative stress as measured by plasma malonylaldehyde (MDA) is also reduced, together with significant inhibition of angiotension converting enzyme (ACE) (Quiñones *et al.*, 2011).

In this current study, the antithrombotic effects of dark chocolate consumption was determined in 22 mild hypertensive patients measured by *ex vivo* analysis of platelet function, as well as study of the *in vitro* effects of theobromine, a major methylxanthine constitutive of cocoa (Smit *et al.*, 2004), on platelet function.

5.2 Methodology

5.2.1 EPICURE Trial

i. Study Design

This study was conducted as a two-phase crossover study of patients recently diagnosed with prehypertension or mild hypertension who were randomised to treatment with high-flavonoid or low-flavonoid dark chocolate for 6 weeks (50g/day) and then swapped over to the alternative treatment for further 6 weeks (50g/day).

ii. Study Group Eligibility Criteria

The eligibility criteria were men with recently diagnosed mild hypertension (i.e. SBP 140 – 159 mmHg; DBP 90 – 99 mmHg) or with constant prehypertension (i.e. SBP 130 – 139 mmHg; DBP 85 – 89 mmHg) assessed by repeated clinic measurements. To exclude patients with white-coat hypertension, 24 hours ambulatory blood pressure was performed during pre-recruitment screening. Individuals recruited to the study were otherwise healthy and free of other disease. This was confirmed by physical examination, 12-lead ECG, and routine blood chemistry including liver function tests. Recruitment was for 32 men that met these criteria.

Listed below are the exclusion criteria and additional criteria for the study:

a. Exclusion criteria:

1) Age <45 years or >70 years
2) Diabetes mellitus or raised fasting blood glucose
3) Total cholesterol >7 mmol/L
4) BMI >30 kg/m^2
5) History of cardiac arrhythmias or ECG abnormalities at baseline
6) History of psychiatric or psychological illness
7) History of hypo- or hyperthyroidism
8) Participation in another trial whether active or in follow-up
9) Abnormal liver function tests or other routine blood chemistry
10) Excessive alcohol consumption
11) Regular medicines for any condition
12) Regular use of herbal medicines or other alternative remedies

b. Additional criteria:

1) No red wine consumption during the study
2) No aspirin or other non-steroidal anti-inflammatory agents for 4 weeks before the study or during the study
3) Allowable pain relief paracetamol

4) Use of any medicines (e.g. antibiotics) to be recorded by the participants during the study for entry into the case report forms.

Every participant was assigned a study number on enrolment. Chocolate bars were supplied coded according to a randomisation scheduled provide by Prof. Atholl Johnston. The investigators and subjects were fully blinded to the treatment schedule.

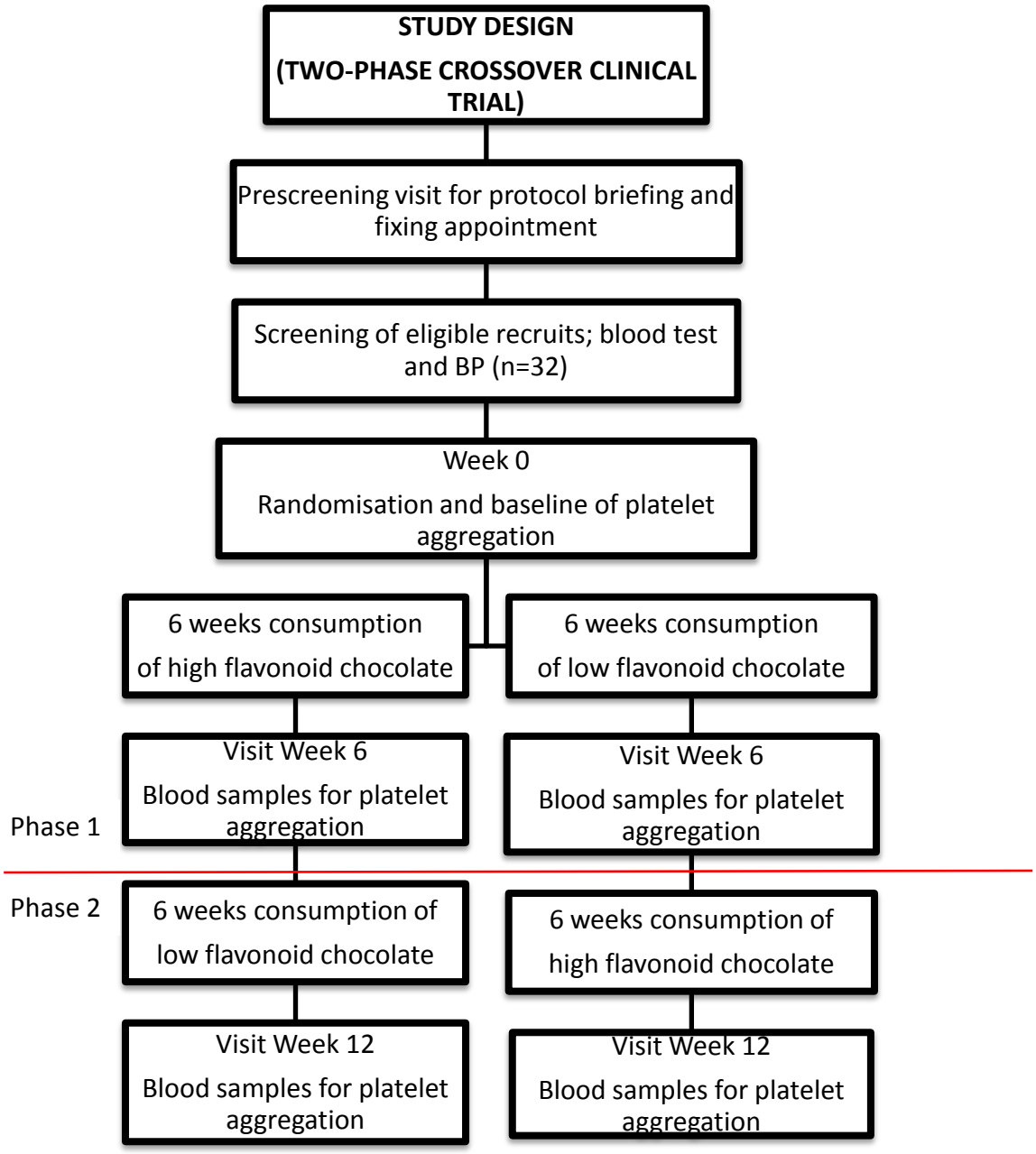


Figure 5.1: *Schematic diagram showing the EPICURE trial.*

5.2.2 Preparation of Wet Agonist Plate

Half-area 96-well plate were used in this study to minimise the PRP required for each measurement. However, wet agonists plates were prepared that could be stored at $4^{o}C$ and used when needed. The agonist final concentrations were the same as previously described in Chapter 3, however, PBS with 0.1% ascorbic acid was used to make up all agonists with arachidonic acid being the last agonist prepared. After all agonists had been added into the wells, plates were vacuum sealed to minimise evaporation or oxidation. Each plate was then labelled with details of date of preparation and batch. Finally, all prepared wet agonist plates were kept at $4^{o}C$ in a cold room. At least 1 plate from each batch was randomly tested for platelet aggregation and adhesion prior to batch usage. Measurement of platelet aggregation and adhesion from this clinical trial was tested as described in previous chapter.

5.2.3 In vitro Platelet Study of Theobromine

The effects of theobromine on platelet function were studied using PRP from healthy volunteers. PRP was incubated with theobromine for 30 minutes prior to addition of platelet agonists. Platelet aggregation and adhesion were then followed as described previously.

5.3 Results

5.3.1 Platelet Investigation in EPICURE Trial

Though there were 32 patients enrolled in this study, only 22 patients successfully completed. Consumption of dark chocolate decreased platelet responses to ADP and TRAP-6 as shown in Figure 5.2-5.7. These effects appeared enhanced with high flavonoid-contained dark chocolate, but no statistical differences were found between low- and high-flavonoid dark chocolate. For example, with respect to ADP-stimulated platelet aggregation, baseline responses at 3, 10 and 30 μM were 12.5±2%, 28.6±2% and 40.7±2%; low dark chocolate, 4.4±2%, 17.7±3% and 31.5±3%; high dark chocolate, 3.9±2%, 18.0±2% and 35.1±2%. Dark chocolate effectively decreased platelet aggregation induced by TRAP-6. At TRAP-6 concentration of 3 μM, platelet aggregation was decreased to 1.0±2% following consumption of low dark chocolate, and 0% by high dark chocolate as compared to control, 24.4±2%, ($p<0.01$). Platelet aggregation in control conditions in response to 10 μM TRAP-6 was 41.0±2%, but was reduced to 20.1±3% in both low and high dark chocolate, ($p<0.05$). At the highest concentration of TRAP-6, 30 μM, platelet aggregation was decreased to 24.2±3% ($p<0.001$) in low dark chocolate and 30.0±2% ($p<0.01$) in high dark chocolate as compared to control, 52.2±2%. However, there were little or no changes in platelet aggregation responses to other agonists.

Platelet adhesion stimulated by ADP and TRAP-6 was also significantly decreased following treatment with dark chocolate (Figure 5.8). For instance, at 10 μM ADP, control platelet adhesion was 28.6±2% but decreased to 20.2±2% in low dark chocolate and 18.2±2% in high dark chocolate. At 30 μM ADP, low dark chocolate reduced platelet adhesion to 30.7±2% and high dark chocolate, 25.3±2% as compared to control, 40.7±2%. Platelet adhesion stimulated by 10 μM TRAP-6 in control (40.4±3%) was decreased in low dark chocolate, 23.9±3% ($p<0.01$), and in high dark chocolate, 19.1±3% ($p<0.001$). At the highest concentration of TRAP-6, 30 μM, platelet adhesion was decreased to 27.6±4% ($p<0.01$) in low dark chocolate and 26.7±3% ($p<0.001$) in high dark chocolate, as compared to control, 46.3±3%.

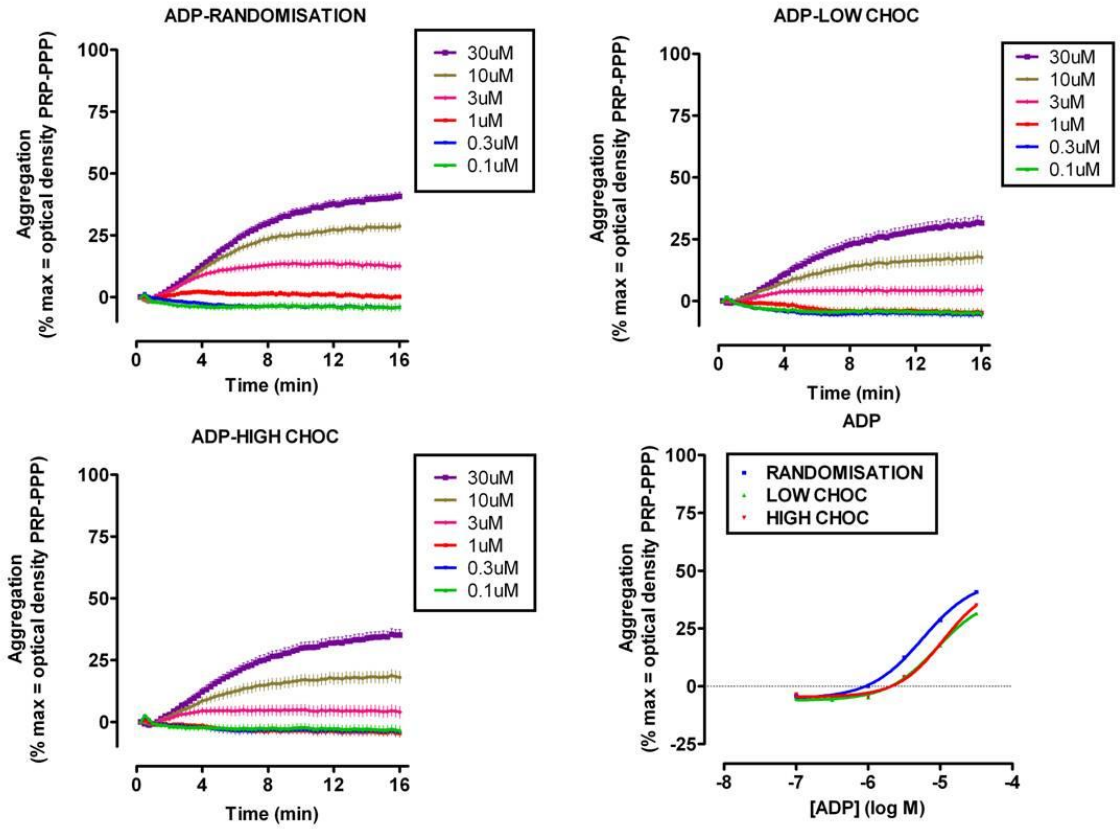


Figure 5.2: *ADP-stimulated platelet aggregation responses in 22 patients with high blood pressure consuming dark chocolate*. Blood samples were collected at randomisation (baseline pre-treatment), and after low and high-flavonoid chocolate. Each value represent means ± s.e (n=22).

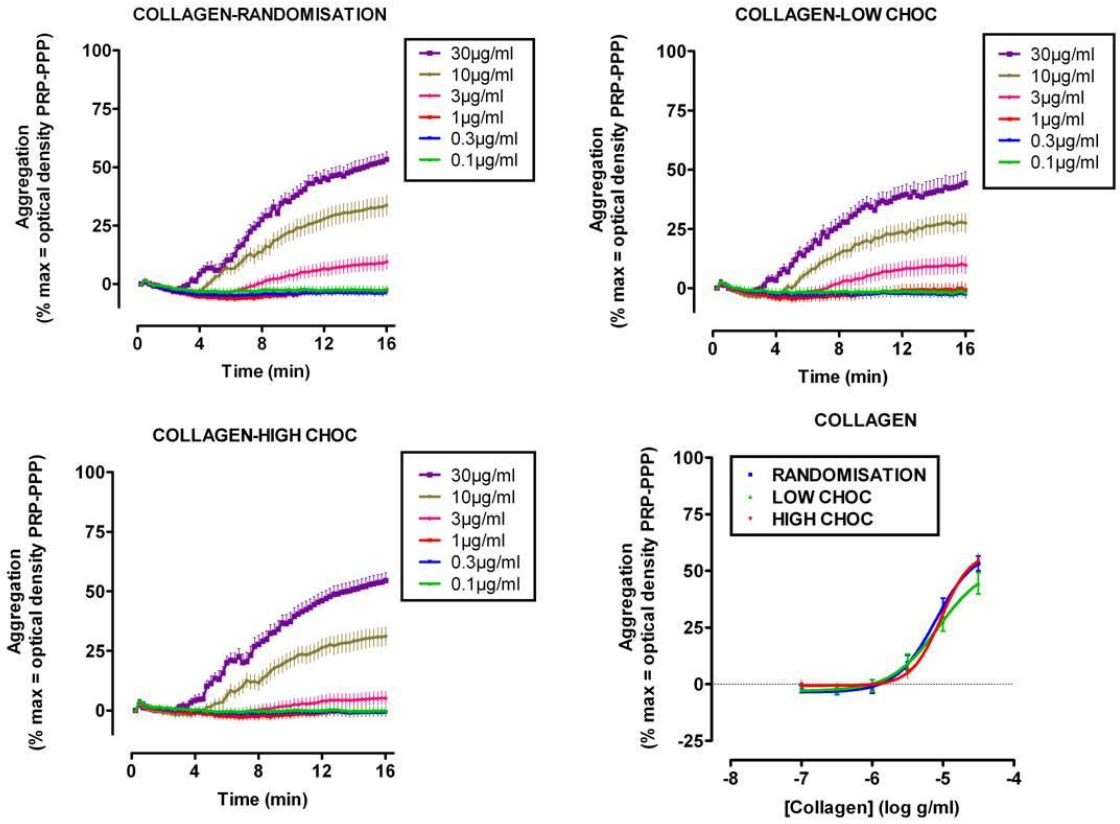


Figure 5.3: *Collagen-stimulated platelet aggregation responses in 22 patients with high blood pressure consuming dark chocolate*. Blood samples were collected at randomisation (baseline pre-treatment), and after low and high-flavonoid chocolate. Each value represent means ± s.e (n=22).

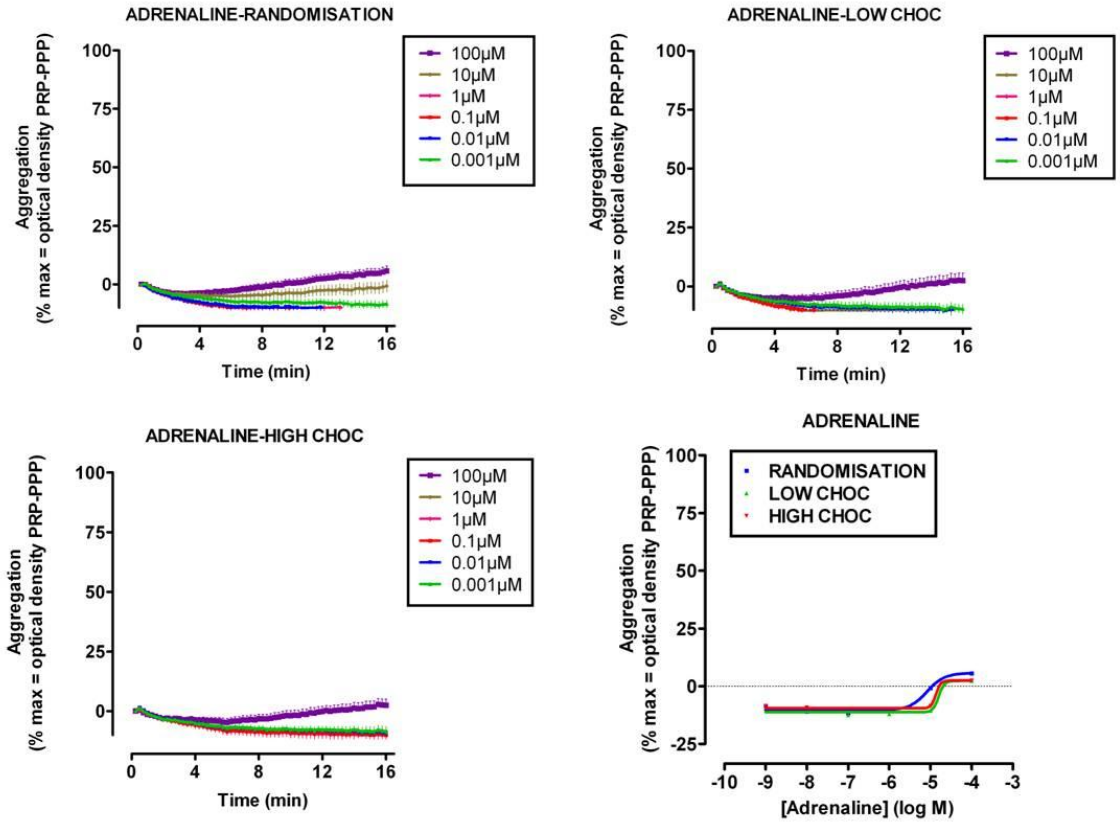


Figure 5.4: *Adrenaline-stimulated platelet aggregation responses in 22 patients with high blood pressure consuming dark chocolate*. Blood samples were collected at randomisation (baseline pre-treatment), and after low and high-flavonoid chocolate. Each value represent means ± s.e (n=22).

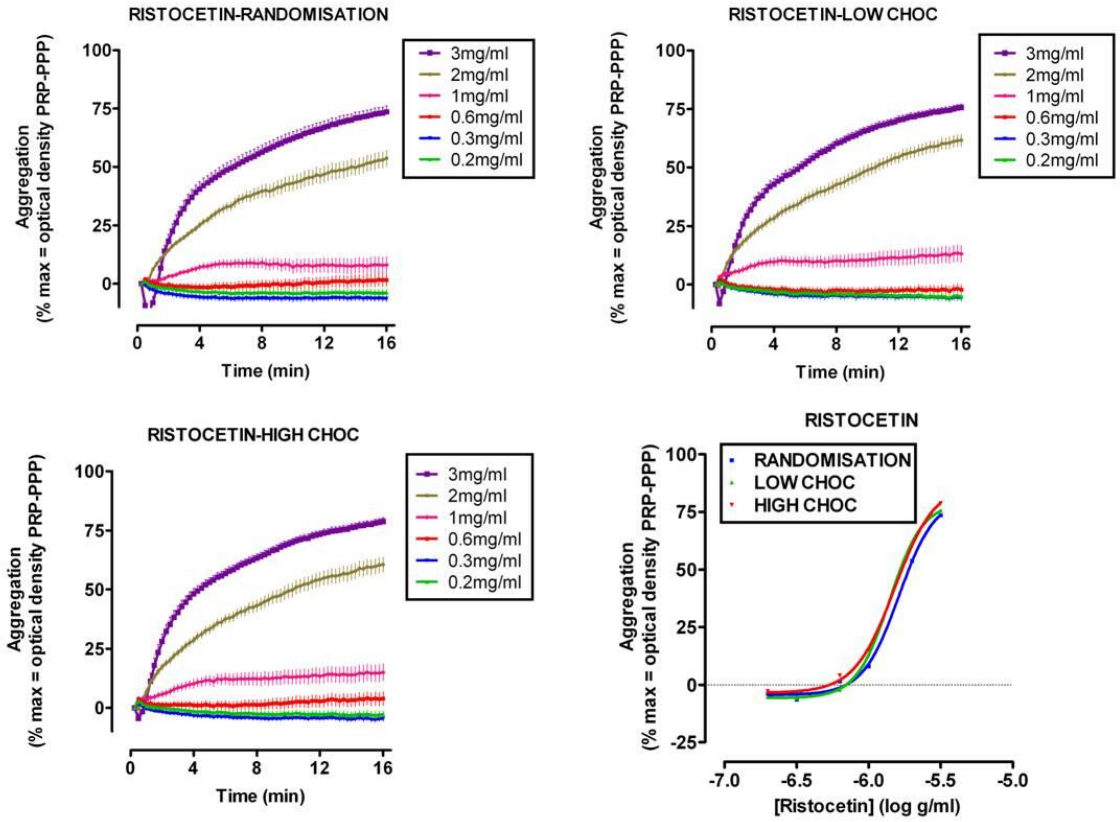


Figure 5.5: *Ristocetin-stimulated platelet aggregation responses in 22 patients with high blood pressure consuming dark chocolate*. Blood samples were collected at randomisation (baseline pre-treatment), and after low and high-flavonoid chocolate. Each value represent means ± s.e (n=22).

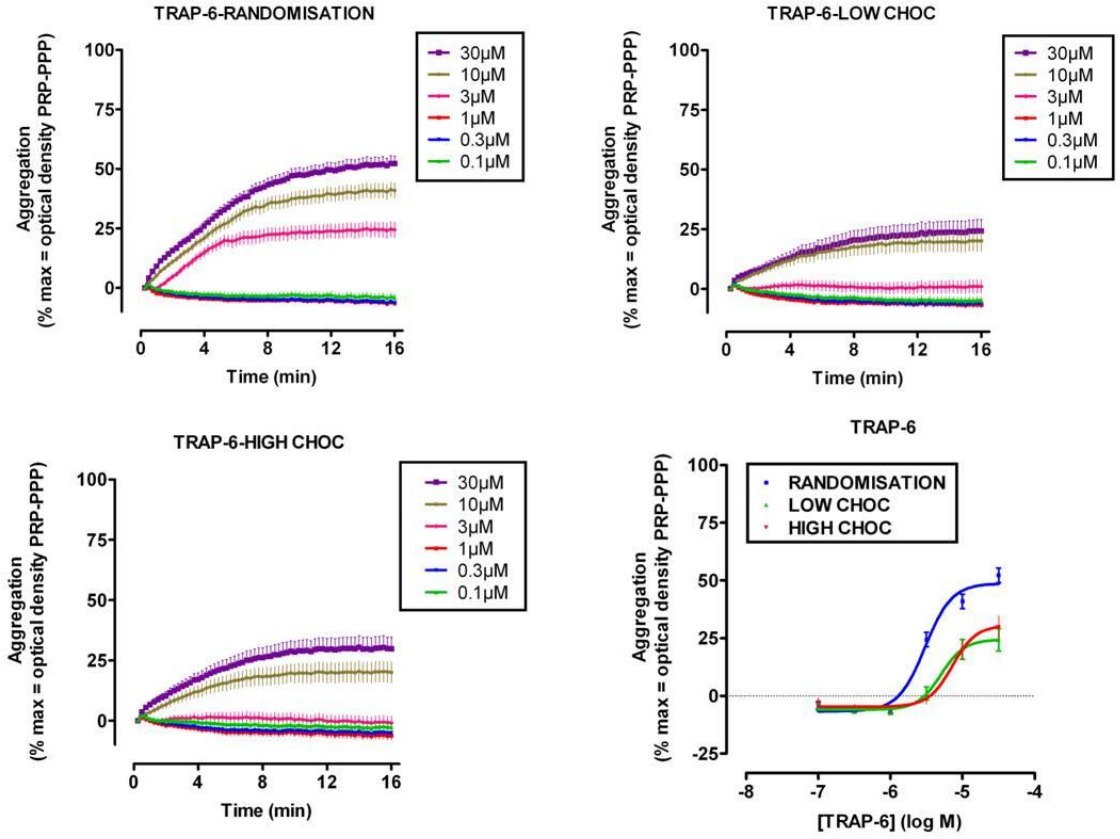


Figure 5.6: *TRAP-6 stimulated platelet aggregation responses in 22 patients with high blood pressure consuming dark chocolate*. Blood samples were collected at randomisation (baseline pre-treatment), and after low and high-flavonoid chocolate. Each value represent means ± s.e (n=22). Concentrations showing significant differences compared to baseline TRAP-6: 3, 10 and 30 µM; LFDC: $p<0.01$, $p<0.05$ and $p<0.001$; HFDC: $p<0.01$, $p< 0.05$ and $p<0.01$ respectively.

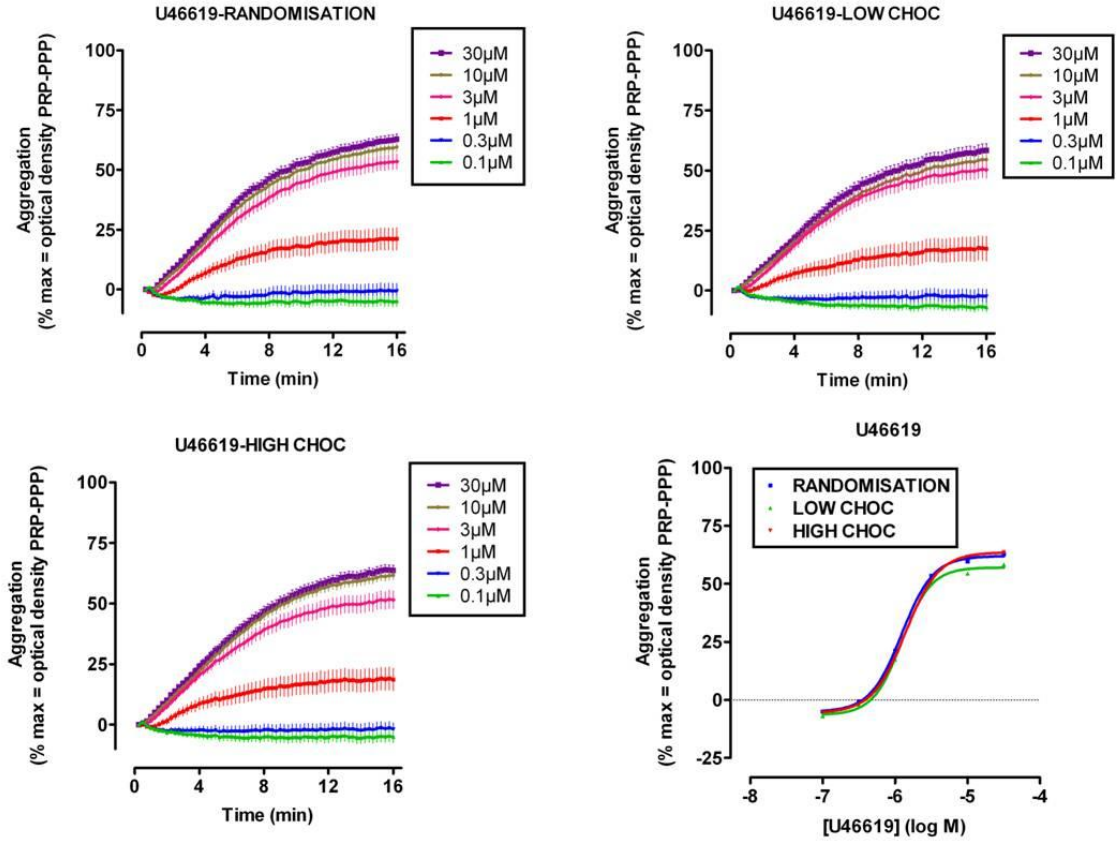


Figure 5.7: *U46619-stimulated platelet aggregation responses in 22 patients with high blood pressure consuming dark chocolate*. Blood samples were collected at randomisation (baseline pre-treatment), and after low and high-flavonoid chocolate. Each value represent means ± s.e (n=22).

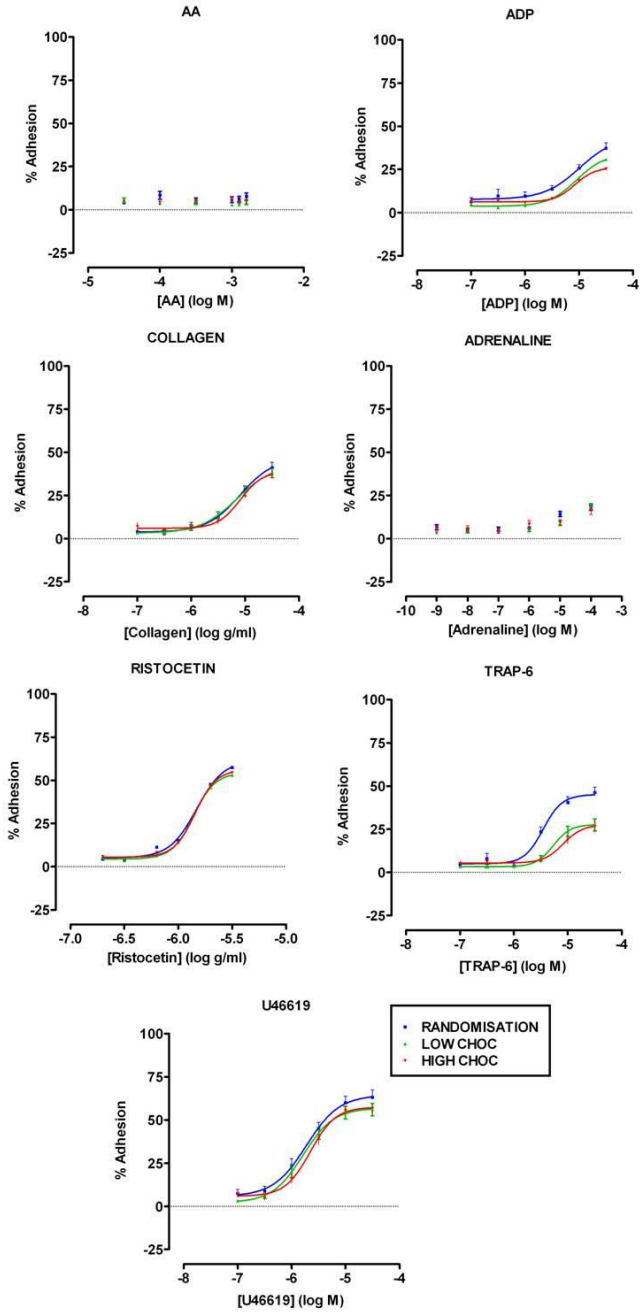


Figure 5.8: *Platelet adhesion responses in 22 patients with high blood pressure consuming dark chocolate*. Blood samples were collected at randomisation (baseline pre-treatment), and after low and high-flavonoid chocolate. Each value represent means ± s.e (n=22). Concentrations showing significant differences compared to baseline TRAP-6: 3, 10 and 30 µM; LFDC: $p<0.01$, $p< 0.05$ and $p<0.001$; HFDC: $p<0.01$, $p< 0.05$ and $p<0.01$, respectively.

5.3.2 Effects of Theobromine, Cocoa Main Components on Platelet Function

Platelet studies performed in the EPICURE Trial showed that both low and high flavonoid dark chocolate inhibited platelet responses to ADP and TRAP-6. Therefore, further studies were conducted to investigate the effects of theobromine, a major compound in cocoa that may responsible for the inhibitory effects of dark chocolate in platelet. In this study, theobromine demonstrated various concentration-dependent inhibitory effects against platelet function (Figure 5.9 and Figure 5.10). For instance, aggregation to ADP 3μM was decreased to 35±9% and 32±7% by 5 and 10μg/ml theobromine, respectively, compared to the vehicle control of 45±9% (n=5). Platelet aggregation induced by 3μg/ml collagen, 69±13%, was also inhibited to 33±14% by 5μg/ml theobromine (n=4). Incubation with theobromine also inhibited platelet aggregation and adhesion induced by arachidonic acid, adrenaline, and TRAP-6 but not that induced by U46619 and ristocetin.

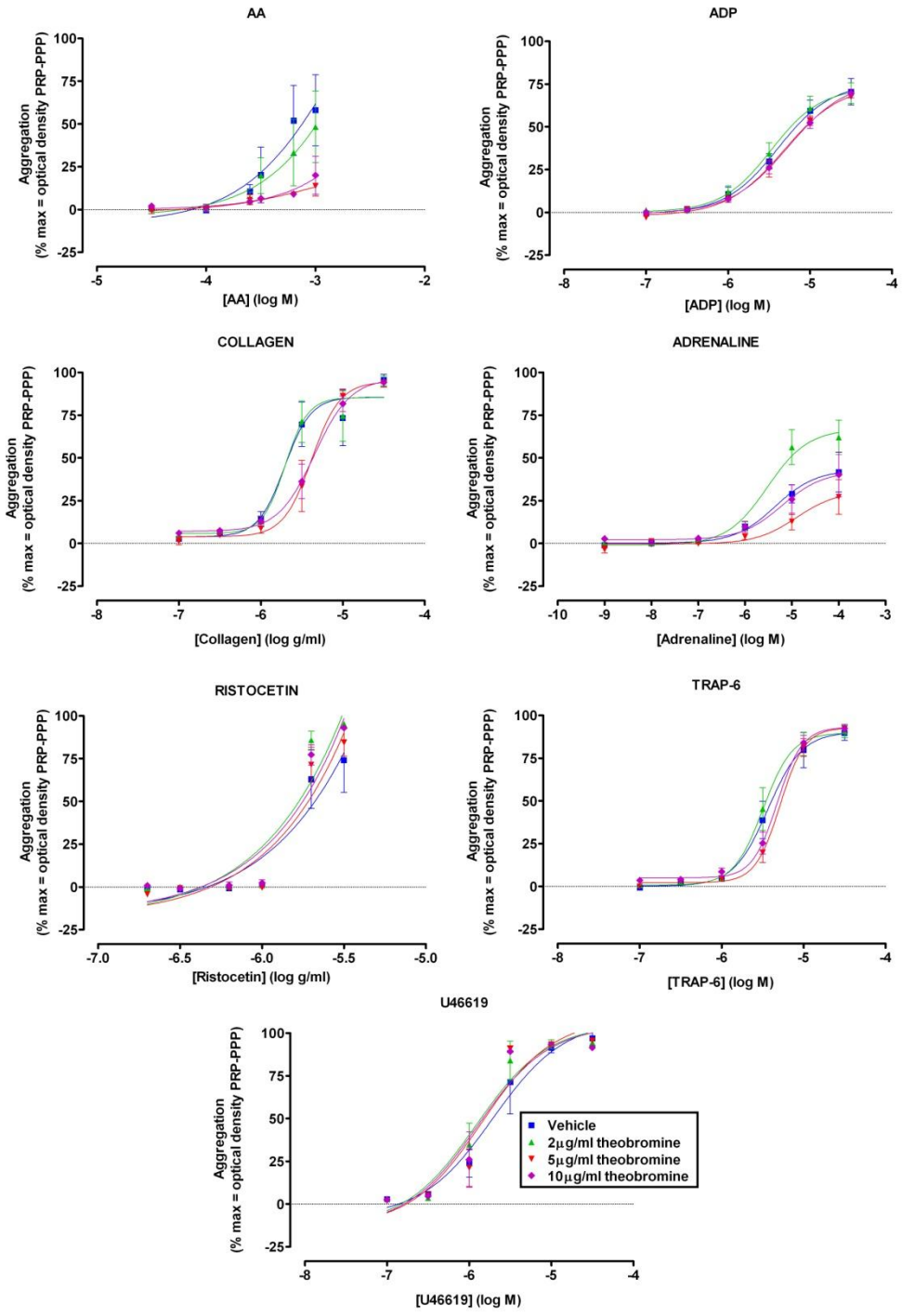


Figure 5.9: *Effects of theobromine on platelet aggregation.* PRP was incubated with theobromine at 2, 5 and 10µg/ml for 30 mins before platelet aggregation was measured in 96-well plate. Each value represent means ± s.e (n=4).

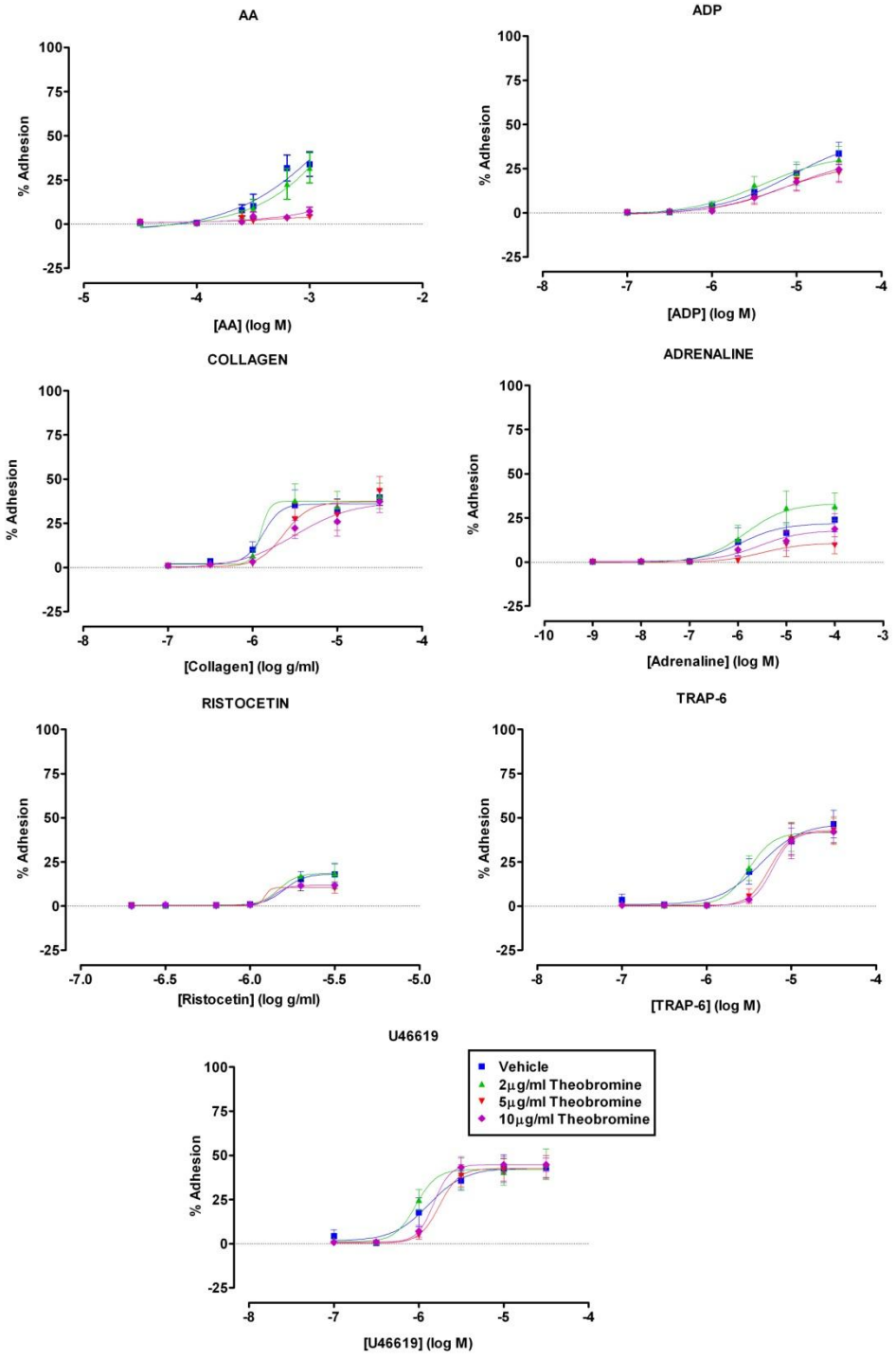


Figure 5.10: *Effects of theobromine on platelet adhesion.* PRP was incubated with theobromine at 2, 5 and 10µg/ml for 30 mins before platelet adhesion was measured in 96-well plate. Each value represent means ± s.e (n=4).

5.3.3 Concentration-Dependent Effects of Theobromine Platelet Responses to ADP and TRAP-6

To determine if the effects of theobromine were concentration-dependent, concentrations of 2-50µg/ml were tested against platelet activation induced by ADP (Figure 5.11) and TRAP-6 (Figure 5.12). Aggregation induced by 3µM ADP was indeed decreased by theobromine in a concentration-dependent manner. For example, aggregation decreased from 45.8±10% in control to 32.6±7%, 33.8±9% and 26.4±7% following incubation with 10, 20 and 50µg/ml theobromine respectively. Platelet adhesion was also inhibited in a concentration-dependent manner; control 9.4±3%, 20µg/ml theobromine 7.6±2%, and 30µg/ml theobromine 5.0±2%. Similar observations were also made regarding TRAP-6-induced platelet activation. For instance, at 2, 10 and 50µg/ml theobromine, platelet aggregation was decreased to 38.0±14%, 35.6±14% and 34.4±15% as compared to control, 51.8±14%. As dark chocolate consumption reduces the ADP and TRAP-6 platelet function in EPICURE trial, this data suggests such an effect may be dependent upon the dose of theobromine that is delivered.

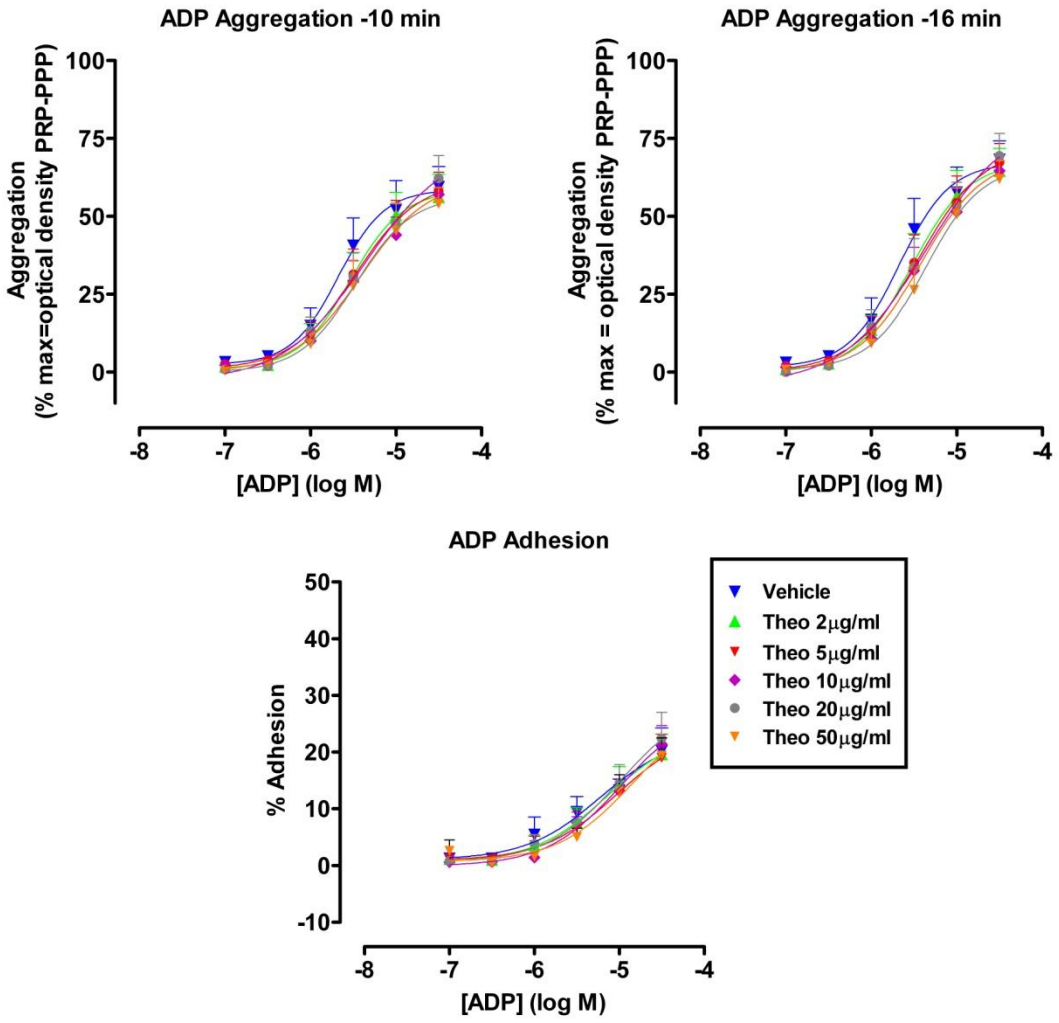


Figure 5.11: *Effects of theobromine on platelet aggregation and adhesion in response to ADP.* PRP was incubated with theobromine at 2, 5, 10, 20 and 50μg/ml for 30 mins before platelet aggregation was measured in 96-well plates. Each value represent means ± s.e (n=5).

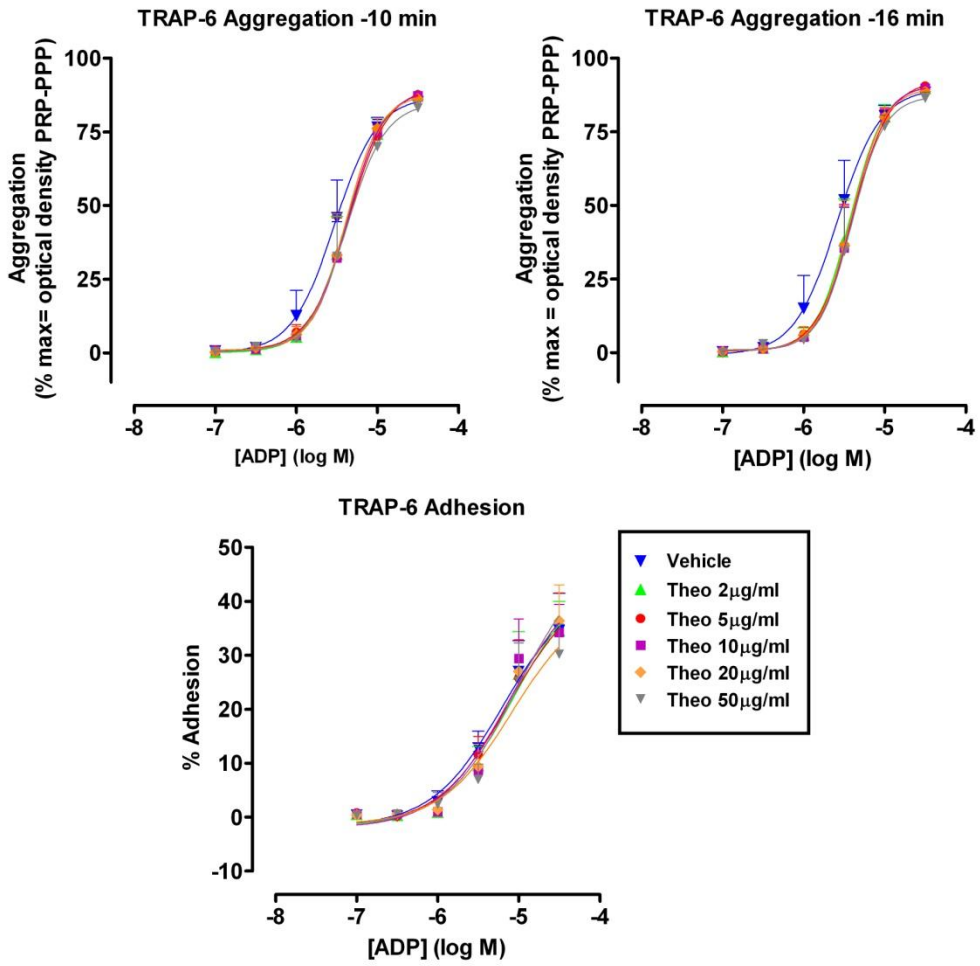


Figure 5.12: *Effects of theobromine on platelet aggregation and adhesion in response to TRAP-6.* PRP was incubated with theobromine at 2, 5, 10, 20 and 50µg/ml for 30 mins before platelet aggregation was measured in 96-well plates. Each value represent means ± s.e (n=5).

5.4 Discussion

The 96-well plate method for platelet aggregation and adhesion determination was expanded to a 'ready-made' wet agonist plate. This allowed the agonist plate to be prepared in advance and so reduced the total test time whenever samples were obtained. For each agonist preparation, 0.1% ascorbic acid in PBS buffer was used to avoid any oxidative reaction and so preserve agonist activity. This method was not used for arachidonic acid because it is unstable and prone to oxidation even if careful precautions were taken. In order to prevent further oxidation and evaporation, the wet agonist plates were vacuum-sealed before being stored at 4^0C. Each batch was then spot tested to confirm all agonists were working.

Even though the effects of dark chocolate on cardiovascular health are still a matter of debate, previous studies have suggested a potential diminished risk of CVD following regular intake of flavonoid-rich food such as dark chocolate. Potential mechanisms underlying this 'aspirin-like effect' of dark chocolate include reduced inflammation, inhibition of atherogenesis by decreased plasma oxidation status, enhancement of endothelial function, inhibition of platelet function to decrease potential thrombosis formation and interference in the production of interleukin 1 beta by peripheral blood mononuclear cells.

Interestingly, the EPICURE study demonstrated that chronic consumption of dark chocolate significantly decreased ADP and TRAP-6 induced platelet aggregation and adhesion in hypertensive patients. This is in line with a previous study that showed inhibition of the ADP and adrenaline-induced platelet activation marker, granular membrane protein CD62P, following acute consumption of cocoa beverage in healthy volunteers (Rein *et al.*, 2000c). Fibrinogen binding with glycoprotein IIb-IIIa is central to platelet activation, but can be inhibited by nitric oxide which increases the intraplatelet levels of cGMP and so reduces agonist-mediated platelet calcium influx. Cocoa beverages inhibit platelet activation by decreasing the fibrinogen-binding conformation of platelet glycoprotein IIb-IIIa (PAC1-positive platelets) and inhibiting the formation of platelet microparticles (Rein *et al.*, 2000c). Furthermore, platelet-related primary haemostasis was delayed as measured by a prolonged closure time in the PFA-100 collagen-epinephrine cartridge, but no changes in the collagen-ADP induced closure time after consumption of cocoa beverage (Rein *et al.*, 2000c).

Cocoa contains a rich mixture of unique flavonoids that has the potential to regulate the immune system and enhance antioxidant activity. The flavonoids in cocoa are mainly catechin and epicatechin which exist in long polymers; for example procyanidins that contain two, three, or up to ten catechin or epicatechin units (Keen, 2001). Cocoa procyanidin has been shown to increase platelet PAC-1 binding and P-selectin expression in vitro. In contrast, cocoa consumption was reported to inhibit platelet activation in healthy volunteers but these effects were not exhibited by dealcoholized red wine or

caffeine consumption (Rein *et al.*, 2000b). One possible reason was the polyphenol content per gram in dark chocolate is higher as compared to other antioxidant-rich sources such as tea, berries or wine ((Hermann *et al.*, 2006a).

From previous studies, it was suggested that dark chocolate would reduce the risk and mortality from cardiovascular disease and could have 'aspirin-like' effects. Consumption of flavonol–rich cocoa decreases the platelet expression of GPIIb-IIIa and P-selectin, with an additive effect when used in combination with aspirin (Pearson *et al.*, 2002). Moreover, cocoa consumption also prolongs platelet-dependent primary hemostasis in both CEPI and CADP closure time in the PFA-100 machine, whilst aspirin only decreased CADP-induced closure time (Pearson *et al.*, 2002). Cigarette smoking is commonly associated with increased risk of coronary heart disease due to endothelial and platelet dysfunction. Nevertheless, consumption of dark chocolate increased the total oxidant status of healthy smokers hence improving endothelial and platelet function, but this effects was not seen white chocolate (Hermann *et al.*, 2006a). In addition, in a study of heart transplantation patients, coronary vasodilatation was increased with improved coronary vascular function and reduced shear stress-dependent platelet adhesion 2 hours after consumption of flavonoid-rich dark chocolate (Flammer *et al.*, 2007). Vascular dysfunctions are often associated with increased oxidative stress and impairment of nitric oxide. The fact that dark chocolate enhance vascular function could be explained by reduction of oxidative stress, activation of nitric oxide synthase and low lipid peroxidation (Flammer *et al.*, 2007).

It is noteworthy that nitric oxide inhibits platelet function by increasing the levels of cGMP, thus stimulation of nitric oxide synthase by dark chocolate attenuates platelet function. The beneficial vascular effects of dark chocolate have been extended to healthy smokers known to exhibit endothelial and platelet dysfunction. These effects were demonstrated by increased flow mediated dilatation measured by ultrasonography of the brachial artery 2 hours after ingestion of dark chocolate. Furthermore, shear stress dependent platelet adhesion was also reduced as total antioxidant status was increased (Hermann *et al.*, 2006a). As cocoa are rich with flavonoids (Keen, 2001), this suggested that the effects of dark chocolate on the pathogenesis of atherothrombosis may rely on the antioxidative effects of dark chocolate flavonoids.

Even though this study demonstrated the beneficial inhibitory effects of dark chocolate consumption in borderline hypertensive patients, no significant differences were found between high and low flavonoids content dark chocolate. Thus, the platelet inhibitory effects of dark chocolate may not be due to the antioxidant effects of flavonoids but may be caused by other compounds such as methylxanthines. Methylxanthines are found in coffee, cocoa products and cola soft drinks. Theobromine (3,7-dimethylxanthine) is a naturally occurring alkaloid that is present in chocolate, tea and cocoa products (Stavric, 1988). Dark chocolate contains higher theobromine, 240-520 mg, as compared to 65-160 mg in milk chocolate, per 50-g portion (MAFF, 1998). It is a metabolite of caffeine, with dark chocolate containing the highest theobromine concentration relative to caffeine, which is present in small amounts in dark chocolate (Smit *et al.*, 2004).

In contrast with caffeine, which has been studied extensively, there is much less research into theobromine. However, there are examples of theobromine beneficial effects associated with cardiac oedema and angina pectoralis, and as a diuretic agent. Moreover, a previous *in vivo* study has shown that theobromine significantly reduces total serum cholesterol, LDL-cholesterol and triglycerides, but elevates significantly HDL-cholesterol (Eteng *et al.*, 2000). Theobromine and caffeine exert stimulant effects on the central nervous system by means of adenosine receptor antagonism, which results in psychopharmacological effects such as improvement of mood and cognitive functions (Smit *et al.*, 2004).

In order to explain the platelet inhibitory effects of dark chocolate from EPICURE trial, *in vitro* effects of theobromine on platelet aggregation and adhesion were determined. Theobromine inhibited the platelet aggregation and adhesion stimulated by ADP, collagen, TRAP-6, adrenaline and arachidonic acid, and these effects were concentration related. These effects may due to inhibition of platelet phosphodiesterase, preventing the conversion of cyclic AMP to AMP and thus inhibiting platelet aggregation. There was limited previous study of other methylxanthines with effects on platelet, but not theobromine. For an example, incubation of washed human platelet with theophylline leads to increased intracellular levels of cAMP, and inhibition of the release of ADP and of lactate production (Wolfe *et al.*, 1970). Since the effects of methylxanthines are related to adenosine, theobromine may have direct antiplatelet effects as adenosine plays a key role

in negative feedback mechanism of platelet activity. In contrast, (Agarwal *et al.*, 1994) suggested that methylxanthines are adenosine receptor antagonists. Nevertheless they have reported that addition of theophylline or caffeine to human PRP with replenished adenosine increased platelet aggregation induced by platelet activating factor. This study also demonstrated the EC_{50} values of PAF in patients receiving chronic treatment of theophylline were significantly lower than those in control subjects (Agarwal *et al.*, 1994).

However, in the case of caffeine, there is a previous report showing that chronic caffeine consumption upregulates adenosine receptors thus decreasing the ability of platelets to aggregate. Choi (2003) showed that *in vitro* treatment of PRP with caffeine reduces platelet aggregation induced by adrenaline and ADP, but does not affect collagen or ristocetin induced platelet activation. Interestingly, in some subjects who are responsive to caffeine, there was loss of secondary wave after exposure to ADP suggesting that caffeine impaired the release of endogenous ADP from platelets (Choi, 2003). Consumption of caffeine and clopidogrel exhibited potential effects in healthy volunteers and coronary artery disease patients (Lev *et al.*, 2007). These effects were seen with platelet aggregation induced by lower concentrations of ADP; decreased platelet surface expression of P-selectin and GPIIb-IIIa receptors and lowered vasodilator-stimulated phosphoprotein (VASP) phosphorylation. The same study also determined the effects of combination of caffeine and clopidogrel in patients with CAD and observed a decrease in P-selectin and PAC-1 binding but no significant effect on aggregation (Lev *et al.*, 2007). Therefore, this suggested that since caffeine causes an increase in cAMP by inhibition of

adenosine receptors, coadministration of clopidogrel and caffeine may have synergistic effects on platelet inhibition. Levels of plasma plasminogen activator inhibitor-1 (PAI-1) are increased in patients with CAD or myocardial infarction and this level is contributed to by high PAI-1 contents in platelets. However, caffeine intake reduces the platelet PAI-1 content following stimulation of platelet release by thrombin (Joerg *et al.*, 1990). From this evidence it could be suggested that theobromine is a platelet inhibitor depending on variations caused by different platelet agonists, and this property may be explained by blockade of the adenosine receptor, possibly A_{2A} receptor, leading to elevations in platelet inhibitory cAMP levels.

Toxicity of theobromine has been reported previously leading to a decreased interest in theobromine research. Rats fed with high amounts of theobromine demonstrated atropy of testes and thymus as well as loss of food intake and reduction in bodyweight (Gans, 1982). Ying *et al.* (1992) demonstrated that theobromine administration in rats alters testicular structure, with vacuolisation of Sertoli cells, abnormal spermatid shape, and impairment of late spermatid release. However, this toxicity effect was reduced with the administration of cocoa-extract that contain equivalent amount of theobromine. The toxicity of theobromine were also influenced by diet, as increased fibre in food tends to decrease its absorption thus reducing the bioavailability of theobromine (Gans, 1982).

Nevertheless, the toxicity of methylxanthines is affected by food intake, particularly over consumption of coffee, strong teas, cola beverages and cocoa products such as dark

chocolate. Whilst theobromine has effects on diuresis, myocardial stimulation, vasodilatation and smooth muscle cell relaxation, it should be noted that its toxicologic effects resulting from combination with other methylxanthines or caffeine metabolites may also limit the potential beneficial effects (Stavric, 1988). In addition, methylxanthines are able to across the placenta and may have disrupt the development of fetus brain. (Wilkinson *et al.*, 1993) shown that with a single maternal dose of caffeine, there were significant dispositions of caffeine and its metabolite, theophylline and theobromine, in fetal and maternal brain. Therefore, it should be noted that administration of methylxanthines especially during pregnancy has to be carefully monitored for any adverse effects.

In conclusion, this study showed clearly that chronic dark chocolate intake inhibits the ability of platelets to aggregate upon stimulation by TRAP-6 or ADP in midline hypertensive patients. However, there is no difference between dark chocolate containing high and low contents of flavonoids, suggesting that it may be methylxanthines such as theobromine that play a role in reducing platelet function. This idea is supported by *in vitro* studies demonstrating that theobromine has various inhibitory effects on platelet aggregation and adhesion. Therefore it is suggested that the data presented here supports previous findings indicating that co-administration of dark chocolate with current cardiovascular disease treatments may supply some additional beneficial effects.

CHAPTER SIX:

GENERAL DISCUSSION

6.1 General Discussion

Platelet function testing is important in clinical settings such as to monitor antiplatelet therapy or prediction of presurgical or perioperative bleeding. Simple point-of-care techniques have been developed and used in clinical settings, but continuous development is still needed to improve platelet function testing. As light transmission aggregometry is still regarded as standard evaluation tool for platelet aggregation, this study aimed at using a modification of traditional Born aggregometry to obtain an uncomplicated, higher output and more reliable method. This modified method involved basic Born aggregometry theory to be adapted to a 96-well plate format to allow higher sample throughput. In addition, the integration of a method to measure adhesion allows additional data to be obtained from the same 96-well plate. Various known platelet inhibitors such as aspirin have been tested using this modified technique with similar results being found as for more established techniques. A further advantage of the 96-well plate format is that it can be adjusted to the need of researchers/clinicians according to their requirement, such as selection of platelet agonists or using a half-volume 96-well plate format to minimise the required blood sample volume. It is noteworthy that standard values have to be developed by individual laboratories for many platelet function test, and this new modified 96-well plate method is without exception. This is because the results from different platelet function tests such as PFA-100, whole blood electrical aggregometry or flow cytometric test of platelet surface antigen are not comparable and not equivalent with each other (Salat *et al.*, 2002).

This study provides additional data regarding the antiplatelet effects of aspirin and other NSAIDs but not of a COX-2 selective inhibitor. The hypothesis that COX-2 selective inhibitors produce a prothrombotic state and so increase cardiovascular adverse effects has to be carefully inferred and investigated reliant upon substantial clinical evidence obtained for this reason. The Therapeutic Arthritis Research and Gastrointestinal Event Trial (TARGET) were designed to compare between COX-2 selective inhibitor, lumiracoxib and NSAIDS, naproxen and ibuprofen in the assessment of gastrointestinal and cardiovascular safety (Farkouh *et al.*, 2004). This large scale study involved more than 18,000 patients with osteoarthritis receiving treatment with lumiracoxib 400mg once daily, naproxen 500mg twice daily or ibuprofen 800mg three times daily for 52 weeks. In this study, researchers found that the primary endpoint which include incidence of myocardial infarction, did not differ between lumiracoxib and either ibuprofen or naproxen, irrespective of aspirin use. Moreover, the post-hoc study analysis of lumiracoxib in the TARGET study did not show any increased risk of developing congestive heart failure when using lumiracoxib compared to NSAIDS (Farkouh *et al.*, 2004). This is supported by Matchaba *et al.* (2005) who found no evidence of lumiracoxib-associated increase of cardiovascular risk compared with naproxen, placebo, or all comparators (placebo, diclofenac, ibuprofen, celecoxib, rofecoxib, and naproxen) through a meta-analysis study of 34, 668 patients receiving $\geq$1 week and up to 1 year of treatment (Matchaba *et al.*, 2005). Moreover, the beneficial effect of lumiracoxib was extended to the reduced risk of GI ulcer complications compared with NSAIDS, naproxen and ibuprofen in patients with osteoarthritis (Hawkey *et al.*, 2007).

My findings in these studies suggest that AA metabolism by platelet COX and LOX are both essential to sustain and regulate platelet functioning in the maintenance of normal hemostasis. Unlike the COX pathway, p12-LOX and its metabolites are much less well understood with regard to influences on platelet function. The contrasting physiological actions of 12(S)-HPETE and 12(S)-HETE suggest that their function is one of the many key regulators of platelet activation. The fact that different biological effects of p12-LOX products were observed in particular platelet activation pathways associated with different agonists, indicates that more in depth research has to be done to study the involvement of these metabolites at molecular level, including cell signalling. Some studies have used washed platelets in the investigation of LOX pathway in platelets, which might influence normal platelet function as washed platelets can be spontaneously activated. I have used PRP in the studies reported here, which contains various plasma proteins including albumin that could interact with AA metabolism and its enzyme activity. Therefore, the methodological differences between researchers could account for the variability of findings. In addition, the lack of biochemical specificity of the p12-LOX inhibitors used should be an important consideration in any interpretation of the data.

Previous reports have suggested that 12(S)-HETE is a down-regulator of AA-dependent pathway platelet activation with 12(S)-HETE being a competitor of the binding of PTA-OH, a thromboxane antagonist, to the thromboxane receptor (TP) (Fonlupt *et al.*, 1991). However, my findings did not detect any inhibition of aggregation and adhesion in platelets challenged with U46619, a thromboxane mimetic agent. Instead, I found that

12(S)-HETE causes an increase in platelet adhesion caused by other agonists such as AA, ADP and adrenaline and increases adrenaline-induced platelet aggregation. Therefore, my conclusion is that 12(S)-HETE is not platelet inhibitor and does not act on TP receptors. In contrast, it may potentiate platelet aggregation by weak agonists through a different platelet signalling pathway which is not clearly understood as yet. Findings in this research also concluded that, in situations in which low concentrations of agonists such as collagen and adrenaline are insufficient to induce high platelet aggregation, amplification of platelet aggregation can be induced by 12(S)-HETE. However, the effect of 12(S)-HETE is limited if p12-LOX is blocked, suggesting that 12(S)-HETE is not as potent as TXA_2 to induce aggregation, which means more 12(S)-HETE is required to be produced from activated p12-LOX. Thus, the inhibition of p12-LOX prevents self-activation of further 12(S)-HETE production through p12-LOX and results in failure to induce platelet aggregation.

My investigations support previous findings that NADPH oxidase is essential for particular agonists to induce platelet activation, such as collagen, ADP and TRAP-6. Therefore, it is suggested that direct inhibition of NADPH oxidase may have potential clinical benefits in preventing thrombotic events among cardiovascular disease patients. However, this assumption is limited by the non-specificity of NADPH oxidase inhibitors, even though emergent research is being done to identify specific inhibitors for NADPH oxidase. In this study, NADPH oxidase inhibitors that are commonly used, DPI and apocynin, were investigated with regard to their effects on platelet aggregation and adhesion. Nevertheless, although DPI is widely used in *in vitro* studies by various researchers, it not

only targets NADPH oxidase enzyme but also inhibits all flavin-containing enzymes, including NO synthases and c-P450 enzymes. It has been established that DPI is a potent NADPH oxidase inhibitor but unlikely to show any selectivity across NADPH oxidase isoforms. For instance, DPI has been shown to effectively inhibit NADPH oxidase activity in various cell types that express one or two isoforms, including endothelial cells (Nox2,Nox4 and Nox1), smooth muscle cells (Nox1 and Nox4), leukocytes (Nox2), fibroblasts (Nox4) and bone marrow-derived hematopoietic stem/progenitor cells (Nox1, Nox2 and Nox4) (Selemidis *et al.*, 2008). The non-specificity of DPI used in this study is overcome by the use of apocynin, which inhibits NADPH oxidase activation by blocking the translocation of p47-phox to the catalytic membrane domain. Apocynin, isolated firstly from the roots of *Apocynum cannabinum* (Canadian hemp.) has been shown to prevent the STZ-induced translocation of p47-phox and p67-phox to the membrane in isolated neutrophils and also to block p47-phox association in endothelial cell membranes. Unlike DPI, apocynin has been postulated to be partly NADPH oxidase isoform selective, based on its mechanism of action. Therefore, apocynin is thought to be more sensitive in inhibiting Nox1, Nox2 and Nox 3 isoforms, which are dependent on the association of cytosolic subunits to the membrane-bound catalytic core upon activation, as compared to other isoforms, Nox4 and Nox5. Thus, as platelets express Nox2 isoforms, it is thought that apocynin effectively inhibits platelet Nox2 since our study demonstrated platelet inhibition after apocynin treatment.

Apocynin has been regarded as the gold standard for selective NADPH oxidase inhibition, despite several lines of evidence showing that its mechanism of action is elusive. It is noteworthy to include previous reports that apocynin activation by myeloperoxidase (MPO) is obligatory for the inhibitory effects of apocynin, which is enhanced following zymosan-induced MPO expression but is inhibited by sodium azide treatment, a MPO inhibitor (Simons *et al.*, 1990). Following that, another study revealed that superoxide anion generation was not inhibited by apocynin in HEK293 cells overexpressing NADPH isoforms (Nox1, Nox2 and Nox4) but interfered with ROS production by other enzymes such as xanthine oxidase (Heumοller *et al.*, 2008). The formation of apocynin dimer, an active form of apocynin by myeloperoxidase was also not found in endothelial cells and smooth muscle cells but detected when myeloperoxidase was supplemented. Therefore, it was suggested that apocynin acts as NADPH oxidase inhibitor in leukocytes, since myeloperoxidase is expressed in these cells, whereas the effects of apocynin in vascular cells is more as an antioxidant (Heumοller *et al.*, 2008). In conjunction to this study, another report has investigated the inhibitory effects of apocynin on washed platelets from normal and NADPH oxidase knockout mice. Collagen and U46619-activated aggregation of washed platelet from male wild type mice C57BL6, Nox2 deficient ($Nox2^{-/y}$) and p47-phox deficient ($p47\text{-}phox^{-/-}$) were effectively inhibited by apocynin (Dharmarajah *et al.*, 2010). Even though the protein expressions of Nox2 and p47-phox are significantly diminished in Nox2 and p47-phox knockout mice, collagen-induced platelet aggregations were similar to those in platelets from normal mice. Based on these investigations, Dharmarajah *et al.* (2010) suggested that the attenuation of platelet aggregation by

apocynin as reported in previous studies is unlikely to be mediated by inhibition of NADPH oxidase activity. Therefore, caution must be taken before any conclusions are made based only upon results from the use of DPI and apocynin. Alternatively, researchers can use other NADPH oxidase inhibitors such as AEBSF (4-(2-Aminoethyl)benzenesulfonylfluoride) which inhibits NADPH oxidase by interfering with the association of p47-phox. AEBSF, however, is not specific as it can also inhibit serine proteases. There is also peptide inhibitor gp91ds-tat, which is specifically designed to inhibit gp91-phox.

Substantial amounts of evidence have been proposed in identifying the generation of ROS by unstimulated and stimulated platelets along with the relationship between ROS and platelet activation. ROS-generating processes other than COX are suggested to be the major source of ROS production in platelets as aspirin does not inhibit ROS formation. Therefore, NADPH oxidase has been identified as one of the pathways in platelets responsible for the ROS formation that may play an important role in platelet function. In addition, platelet 12-LOX also has been suggested to be another important source of ROS in platelets as it catalyzes the formation of hydroperoxide. The importance of ROS in platelet activation has been investigated by looking at platelet aggregation and serotonin release induced by platelet-produced superoxide anions (Handin *et al.*, 1977). Further, association of platelet ROS production and platelet activation has been suggested as a result of studies looking at PLA_2 stimulation by ROS to enhance the liberation of AA (Hashizume *et al.*, 1991). Another study also supports the involvement of ROS in the activation of PLA_2, with DPI inhibiting PLA_2 activation and ROS formation in a

concentration-dependent manner (Goldman *et al.*, 1992). ROS are proposed to exert their effects on platelets by facilitating Ca^{2+} mobilization, activation of protein tyrosine kinase (PTK) or reaction with platelet NO, but more lines of evidence are needed to support this. A recent study has explored ROS-dependent GPVI activation using convulxin, a specific GPVI-selective agonist, and thrombin-treated platelets (Bakdash *et al.*, 2008). It was showed that convulxin induces platelet intracellular ROS production, whilst ROS are released extracellularly in thrombin treated platelets. In the same study, platelet activation induced by both platelet agonists was attenuated by antioxidants including DPI and apocynin, but not by extracellular addition of the antioxidant superoxide dismutase (SOD). Thus, it can be inferred that the role of ROS in platelet activation is dependent on both particular agonists and the localisation of ROS formation. The autocrine and paracrine signalling of platelets by intracellular and extracellular release of platelet ROS is crucial during the interaction between platelets, leukocytes and endothelial cells in the pathogenesis of thrombosis. The extracellular ROS production from activated platelets and leukocytes enhances platelet adherence and recruitment to facilitate thrombus formation. However, the release of platelet substances such as serotonin, SOD and peroxidise will block ROS production by leukocytes, therefore inhibiting further stimulation from platelet-leukocyte interactions. This is important in normal hemostasis and the prevention of vascular plugs that lead to thrombotic diseases. As several studies have reported the involvement of LOX and NADPH oxidase pathways in the formation by platelets of ROS and peroxides, the effects on platelet aggregation and adhesion of inhibition of both pathways were assessed *in vitro*.

Clinical investigations of dark chocolate on platelet function in baseline hypertensive patients suggested the potential of dark chocolate as an anti-thorombotic. This was supported by *in vitro* data in this study showing that theobromine, an antioxidant found in cocoa products, attenuated platelet aggregation and adhesion. Although there is mounting evidence of platelet activation modulation by antioxidants, their effects on platelet function in vivo remains controversial. Despite this, nutritional antioxidants continue to be an area research interest regarding possible antiplatelet and antithrombotic agents. For instance, resveratrol (*trans*-3,4'5-trihydroxystilbene) that is naturally contained in red wine, inhibits aggregation of washed platelets and PRP in response to ADP, collagen and TRAP-6 (Sobotková *et al.*, 2009). In addition, trolox, a vitamin E analogue, was also found to be as potent an antiaggregatory agent as resveratrol in this study. However, trolox is not as potent as resveratrol in inhibiting platelet COX-1 and TXB_2 production, suggesting that its antioxidant effects on ROS-induced platelet activation is targeted at a specific pathway.

There are several studies of nutritional antioxidants with potential antiplatelet effects. One of the studies reported a black soybean extract with potent inhibitory effects on collagen-induced platelet aggregation and serotonin secretion (Kim *et al.*, 2011). In addition, black soybean was also found to attenuate thrombus formation in a $FeCl_3$-induced rat venous thrombosis model. Interestingly, another study was done to investigate the biological properties of milk produced by cows, goats and donkeys (Simos *et al.*, 2011). In this study, of all milk tested, goats' milk showed the highest total

antioxidant capacity *in vitro* and *in vivo*. Goats' milk also reduced platelet aggregation when ADP or PAF was used as agonists in *ex vivo* experiments using PRP from healthy volunteers. However, at the same concentration of goats' milk that caused 100% inhibition of platelet aggregation, milk from cows and donkeys had no inhibitory effects against platelets. Therefore, it could be concluded that the antiplatelet effects of nutrition are strongly related to the antioxidant properties in the food itself. Other studies looking at antiplatelet effects of antoxidants include wines (Pace-Asciak *et al.*, 1996), olive phenolic compound (Zbidi *et al.*, 2009), and herbal medicines such as *Hippophae rhamnoides* (Cheng *et al.*, 2003).

Based on *in vitro* and *in vivo* data in this research, I have concluded that our new modified technique of measuring platelet aggregation in 96-well plate is sufficiently reliable to be used in research laboratories or in clinical settings. I have also showed that other than COX-1, platelet 12-LOX plays a role in controlling platelet function, with 12(S)-HETE as a positive regulator of platelet activation. In addition, my experiments also investigated the involvement of NADPH oxidase in platelet function, and suggested that platelet stimulation by ADP, adrenaline, collagen and TRAP-6 induces formation of active NADPH oxidase complex, either direct or indirectly in the platelet activation pathway. I have summarised the proposed mechanism of platelet activation by platelet 12-lipoxygenase (12-LOX) pathways and NADPH oxidase in Figure 6.1. Finally, another aspect of antiplatelet influences from nutritional sources has been studied in the *in vivo* trial of dark chocolate that could provide a future prospect for research into new drug development.

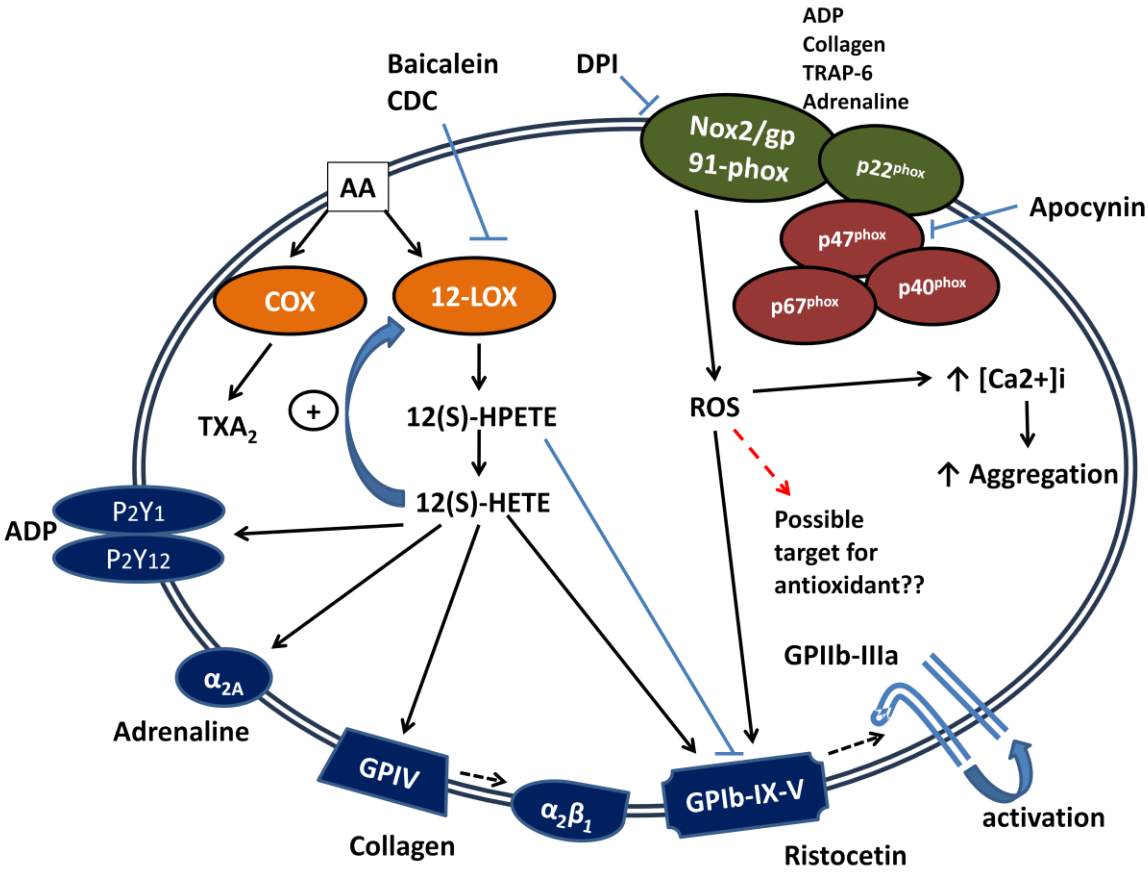


Figure 6.1: *Proposed mechanism of platelet activation by platelet 12-lipoxygenase (12-LOX) pathways and NADPH oxidase.* Arachidonic acid, AA is metabolised by cyclooxygenase enzyme (COX) to produce thromboxane A_2 (TXA_2) which is an important platelet activator. AA is also metabolised by 12-LOX to produce 12(S)-HPETE and then 12(S)-HETE. 12(S)-HPETE acts as platelet inhibitor which may inhibit GPIb-IX-V and so inhibit ristocetin-induced platelet activation. This is in contrast with 12(S)-HETE, which may promote platelet activation through ADP, adrenaline, collagen and GPIb-IX-V receptors and initiate further 12(S)-HETE production by 12-LOX. Upon stimulation by collagen, ADP, TRAP-6 and adrenaline, NADPH oxidase complex will form by the assembly of intracellular components (p47-phox, p67-phox and p40-phox) with the membrane-bound component (NOX-2, p22-phox) resulting in the formation of reactive oxygen species (ROS). Intracellular ROS formation will stimulate the GPIb-IX-V receptor and then activate the GPIIb-IIIa receptor. ROS could be a target for antiplatelet influences from nutritional antioxidants.

CHAPTER SEVEN:

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Appendix 1: List of materials used in this study

[H3] Thromboxane B_2 Tracer	GE Healthcare, UK
[I-125] PGE_2 tracer	Perkin Elmer, USA
12(S)-HETE	Cayman Chemical Company, USA
12(S)-HPETE	Cayman Chemical Company, USA
4-Nitrophenyl phosphate disodium salt hexahydrate	Sigma, UK
Acetylsalicylic acid (aspirin)	Sigma, UK
Activated charcoal	Sigma, UK
Adenosine diphosphate	Labmedics, UK
Albumin	Sigma, UK
Arachidonic acid (peroxide free)	Cayman Chemical Company, USA
Apocynin	Sigma, UK
Baicalein	Sigma, UK
C57Black6 mice	Harlan, UK
Calcium ionophore	Sigma, UK

CDC (cinnamyl-3,4-dihydroxy-α-cyanocinnamate)	Biomol Research Lab, USA
Citric acid	Sigma, UK
Collagen	Labmedics, UK
Dextran	GE Healthcare, UK
Diclofenac	Sigma, Poole, UK
Diclofenac sodium (Voltarol®)	Novartis, UK
Disodium hydrogen phosphate	Sigma, UK
Epinephrine (Adrenaline)	Labmedics, UK
Fibrinogen (Fraction I, Type I from human plasma)	Sigma, UK
Glucose	Sigma, UK
Heparin	CP Pharmaceuticals Ltd, UK
HEPES	Sigma, UK
Lipopolysaccharides	Sigma, UK
Lumiracoxib	Novartis, UK
Magnesium chloride	Sigma, UK

Parecoxib sodium (Dynastat®)	Pfizer, USA
Phosphate buffered saline (PBS)	Sigma, UK
Potassium chloride	Sigma, UK
Prostacyclin (PGI_2)	Sigma, UK
Ristocetin	Helena Biosciences Europe
Rosiglitazone	Sigma, UK
Saline	Baxter, UK
Scintillation fluid (Ultima Gold)	Perkin Elmer, USA
Sodium carbonate	Sigma, UK
Sodium chloride	Sigma, UK
Sodium hydroxide	Sigma, UK
Sodium nitroprusside	Sigma, UK
Thrombin	Chronolog, USA
TRAP-6-amide	Bachem, UK
Tri-Sodium citrate	Sigma, UK
Triton-X-100	Sigma, UK

Trizma base	Sigma, UK
U46619	Cayman Chemical Company, USA
Urethane	Sigma, UK
Theobromine	Sigma, UK

Appendix 2: Composition of Buffer

Dilution Buffer:

- 145mM NaCl
- 5mM KCl
- 10mM HEPES
- 0.5Mm $NAHPO_4$
- 6mM glucose
- 0.2% human serum albumin

Tyrodes Buffer:

- 134mM NaCl
- 2.9mM KCl
- 1mM $MgCl_2$
- 0.34mM Na_2HPO_4
- 12mM $NaHPO_3$
- 20mM HEPES
- 5mM glucose

(pH 7.3)