The relationship between cyclo-oxygenase isoforms and platelets in the cardiothoracic system

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Declaration of originality

I declare that the work reported in this thesis is all my own, with the following exceptions:

- Platelet aggregation experiments reported in Chapter 3 were performed jointly with Dr. Paul C.J. Armstrong, Department of Translational Medicine and Therapeutics, William Harvey Research Institute, Queen Mary University of London. This is reflected in the shared first authorship of the publication derived from this work (see page 14).
- Platelet aggregation experiments reported in Chapter 4 were performed with Dr. Nicholas S. Kirkby, Department of Cardiothoracic Pharmacology, National Heart and Lung Institute, Imperial College London.
- The quantificationss of urinary prostanoid metabolites reported in Chapter 5 were performed by Prof. Ginger Milne and Ms. Sarah Thomas in the Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN, USA.
- The metabolomic analyses of urine samples reported in Chapter 5 were performed by Metabolon Inc., Durham, NC, USA.

Abstract

At present, the cyclo-oxygenase inhibitor aspirin and the $P2Y_{12}$ receptor blocker clopidogrel are commonly combined to prevent recurrent thrombosis after acute coronary syndrome events. Variable responses to this drug combination have driven the development of prasugrel and ticagrelor, two more potent $P2Y_{12}$ blockers that constitute a superior therapy when used with aspirin. In parallel, investigations of non-steroidal anti-inflammatory drugs – the drug class that aspirin belongs to – suggest that cyclo-oxygenase inhibition can contribute to thrombosis.

This thesis has investigated the relationship between cyclo-oxygenase inhibition within the cardiothoracic system and pathways of platelet activation.

It has been demonstrated *in vitro* that the effects of aspirin vary with the level of $P2Y_{12}$ blockade: at low levels of $P2Y_{12}$ inhibition aspirin increases the overall anti-platelet effect, although with high levels of $P2Y_{12}$ blockade aspirin has little to no effect. By giving healthy volunteers prasugrel it was determined that these *in vitro* observations hold true in man – aspirin added little to the anti-platelet effect of prasugrel and furthermore aspirin reduced urinary metabolites of the anti-thrombotic hormone prostacyclin. These data suggest that concomitant use of aspirin with strong $P2Y_{12}$ blockers could lessen the overall anti-thrombotic effect.

Whilst initial experiments used 96-well plate assays to model platelet activation, responses in traditional light transmission aggregometry were also investigated. By investigating parameters affecting the pharmacology of $P2Y_{12}$ blockers and aspirin, it has been possible to ascertain key differences in the way these techniques model platelet aggregation.

In addition, immunoblotting and functional studies of tissues from wild type and COX-1 or COX-2 deficient mice suggest that COX-1 is the predominant isoenzyme found within the cardiothoracic system.

Overall, this thesis questions the therapeutic usefulness of aspirin in combination with potent $P2Y_{12}$ receptor blockers and provides a basis for future work to redefine the role of aspirin in clinical populations.

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Publications

The work presented in this thesis has contributed to the following publications:

Papers

Kirkby N.S., Leadbeater P.D.M., Chan M.V., Nylander S., Mitchell J.A., Warner T.D. Anti-platelet effects of aspirin vary with the level of $P2Y_{12}$ receptor blockade supplied by either ticagrelor or prasugrel *J Thromb Haemost* 2011; **9**: 2103-05

Leadbeater P.D.M., Kirkby N.S., Thomas S., Dhanji A.R., Tucker A.T., Milne G., Mitchell J.A. & Warner T.D. Aspirin has little additional *ex vivo* anti-platelet effects in healthy volunteers receiving prasugrel *J Thromb Haemost* 2011; **9**: 2050-56

Armstrong P.C.J.*, Leadbeater P.D.M.*, Chan M.V., Kirkby N.S., Jakubowski J.A., Mitchell J.A. & Warner T.D. In the presence of strong $P2Y_{12}$ receptor blockade, aspirin provides little additional inhibition of platelet aggregation. *J Thromb Haemost* 2011; **9**: 552-61
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Abstracts

Leadbeater P.D.M., Farnborough R., Mitchell J.A. and Warner T.D. The effect of stir-speed on GPVI-dependent platelet aggregation *J Thromb Haemost* 2011; **9**: Suppl 2

Leadbeater P.D.M., Mitchell J.A. and Warner T.D. The effect of stir-speed and time on TP-receptor induced platelet aggregation in the presence of aspirin and $P2Y_{12}$ receptor blockade *J Thromb Haemost* 2011; **9**: Suppl 2

Leadbeater P.D.M., Mitchell J.A. and Warner T.D. The effect of time on platelet aggregation assessed by light transmission *J Thromb Haemost* 2011; **9**: Suppl 2

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Philip D. M. Leadbeater, Paul C. J. Armstrong, Nicholas S. Kirkby, Melissa V. Chan, Joseph A. Jakubowski, Jane A. Mitchell & Timothy D. Warner. *In vitro* aspirin has little additional anti-platelet effect in the presence of prasugrel active metabolite. *pA2 online 2010*; **8**(1) abst 146P.

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Abbreviations

AA	Arachidonic Acid
ACS	Acute Coronary Syndrome
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
ARU	Aspirin Reactivity Unit
ASA	Aspirin; acetylsalicylic acid
ATP	Adenosine Triphosphate
BCAA	Branched Chain Amino Acid
BSA	Bovine Serum Albumin
C	Celsius
cAMP	cyclic Adenosine Monophosphate
COX	Cyclo-oxygenase
CRP	Collagen-related peptide
CT	Closure Time
CYP450	Cytochrome P 450
DMSO	Dimethyl Sulfoxide
EIA	Enzyme-linked Immunoassay
GP	Glycoprotein
HTRF	Homogeneous Time-Resolved Fluorescence
IC	Inhibitory Concentration
IgG	Immunoglobulin G
LPS	Lipopolysaccharide
LTA	Light Transmission Aggregometry
NSAID	Non-Steroidal Anti-Inflammatory Drug
o.d.	once daily
PAM	Prasugrel Active Metabolite

PAR	Protease Activated Receptor
PBS	Phosphate Buffered Saline
PCI	Percutaneous Coronary Intervention
PFA	Platelet Function Analyser
PG	Prostaglandin
PGI-M	2,3-dinor-6-keto-$PGF_{1\alpha}$
PMSF	Phenylmethansulfonyl Fluoride
PPAR	Peroxisome Proliferator-Activated Receptor
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
rpm	revolutions per minute
s.e.m.	standard error of the mean
TCA	Tri-Citric Acid
TRAP	Thrombin Receptor Activating Peptide
TX	Thromboxane
TX-M	11-dehydro-TXB_2
VASP	Vasodilator-Stimulated Phosphoprotein
vWF	von Willebrand Factor

Chapter 1: Introduction

1.1 Cyclo-oxygenases and prostanoids

The two main members of the cyclo-oxygenase (COX) family present in man are COX-1, encoded by the *Ptgs1* gene on chromosome 9 [1], and COX-2, encoded by the *Ptgs2* gene on chromosome 1 [2]. Although not genetically linked, COX-1 and -2 are structurally and functionally very similar, with approximately 60% homology in amino acid sequence [2]. The primary function of the COX enzymes is to generate prostaglandin H_2 from arachidonic acid [3], which involves both a cyclo-oxygenase and a peroxidase active site contained within the enzymes' large catalytic domains [4, 5]. The function of the COX isoforms explains their alternative name, prostaglandin endoperoxide H synthase (PGHS)-1/-2. From prostaglandin H_2 as a precursor, a number of prostanoids, which are biologically active lipid mediators, are formed and the processes involved in this are outlined below.

1.1.1 COX isoforms: structure, regulation and expression

The *Ptgs1* gene has a structure that is described as typical of a housekeeping gene [1], containing few regulatory elements leading to the assumption that the widespread expression of COX-1 protein is constitutive [3]. However, *Ptgs1* transcription may be regulated, for instance by the developmental transcription factor Sp1 [6].

The confirmation of a second COX isoform was preceded by the observation that certain stimuli, such as lipopolysaccharide and interleukin 1β could induce COX activity and the later discovery that a distinct COX gene was induced [7]. Sequencing of the *Ptgs2* gene identified the inclusion of many regulatory elements within its structure, consistent with early response genes, which undergo dynamic regulation by extracellular stimuli and changing cellular environments [2]. Specific stimuli for COX-2 induction include growth factors, pro-inflammatory cytokines and bacterial

products [8]. In addition to being induced by acute stimuli, COX-2 is also found in some tissues including the kidney and brain under normal conditions, suggesting either constitutive expression or tonic stimulation of COX-2 induction.

Whilst there are many genetic differences between the two isoforms the COX-1 and COX-2 proteins are structurally and functionally similar, although amino acid substitutions in the active site region do have interesting pharmacological consequences, as detailed later in this chapter [9].

1.1.2 Generation of prostanoids

The generation of prostanoids requires the concerted actions of at least two enzymes to generate PGH_2, a precursor that undergoes further enzymatic and non-enzymatic conversion to form a number of distinct lipid mediators [9], as shown in Figure 1.1. The primary – and rate limiting – step is the liberation of arachidonic acid from plasma membranes by the actions of secretory and cytosolic phospholipases A_2 [3]. These enzymes hydrolyse glycerophospholipids at the sn-2 position to release arachidonic acid [10]. Free arachidonic acid interacts with two distinct areas in the large active site of both cyclo-oxygenase isoforms to first of all catalyse the formation of PGG_2, in a cyclo-oxygenase reaction, and then convert this intermediate, by peroxidation, to PGH_2 [3]. The mechanisms of catalysis rely upon stereochemically controlled removal of a hydrogen ion then oxidation by free radicals to generate a cyclic peroxide that is reduced to PGG_2 [11]. Two residues central to this process appear to be Tyr-385, which assists in electron transfer during the oxidation step and Arg-120, which supports arachidonic acid binding to the COX active site [11]. From PGH_2, the prostaglandins PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2 (prostacyclin) and thromboxane A_2 (TXA_2) are formed [3]. The conversion of PGH_2 to PGE_2 can occur

non-enzymically, although to form all other prostanoids the concerted actions of specific synthase enzymes are required [figure 1.1].

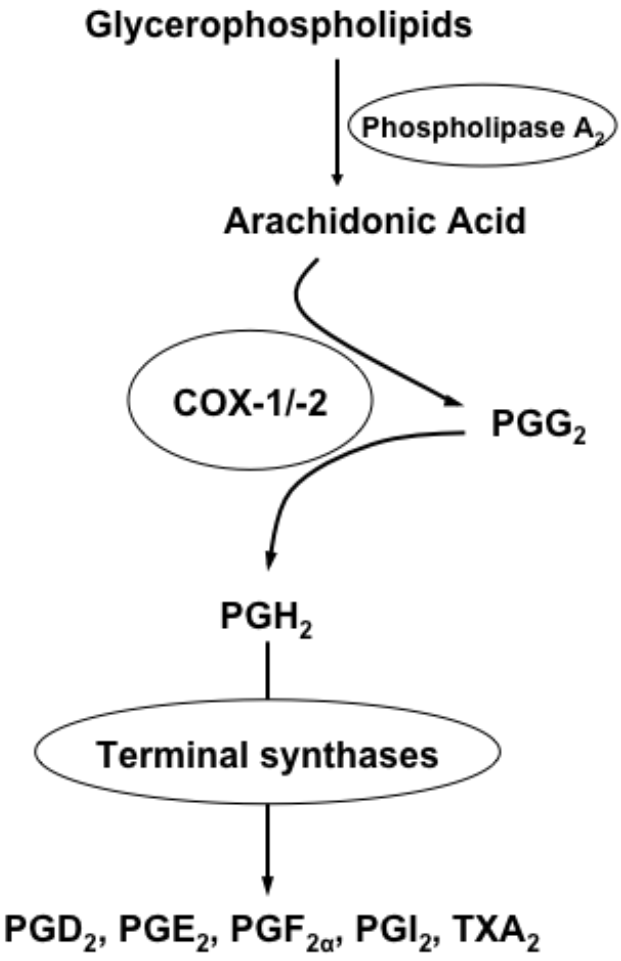


Figure 1.1 The prostanoid pathway

Prostanoids are derived from arachidonic acid, which is liberated from glycerophospholipids by the phospholipases A_2. The COX-1/-2 isoenzymes catalyse the oxidation of arachidonic acid to the prostaglandin endoperoxide PGG_2 and this intermediate is reduced by peroxidation to PGH_2. The actions of specific terminal synthases regulate the formation of the prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2.

1.1.3 Prostanoids in the cardiothoracic system

Prostacyclin

Prostacyclin (PGI_2) is generated from PGH_2 by the enzyme prostacyclin synthase [12, 13]. PGI_2 has a short half-life (2-3 minutes) in the circulation [14] and forms the stable metabolite 6-keto-$PGF_{1\alpha}$, which can be measured as a surrogate of PGI_2 formation *in vitro* and *ex vivo*. The effects of PGI_2 are signaled through the G-protein coupled prostacyclin (IP) receptor, which couples to G_s and when activated signals an increase in adenylyl cyclase activity thus increasing intracellular cAMP. In the vasculature, prostacyclin synthase is concentrated in the endothelium [15, 16]. PGI_2 diffusing across the basal surface of endothelial cells acts on IP receptors on the underlying smooth muscle to signal smooth muscle relaxation [7], whereas PGI_2 that diffuses across the luminal surface binds to platelet IP receptors, inhibiting platelet activation and aggregation [7, 13]. In the cardiovascular system PGI_2 is an anti-inflammatory, anti-thrombotic and anti-angiogenic factor and can be considered a protective homeostatic mediator [7].

Thromboxane A_2

In 1975 Hamberg et al. identified a rapidly metabolised platelet-derived prostanoid with pro-aggregatory properties as thromboxane A_2 (TXA_2) [17]. This had a pharmacological profile identical to that of the previously identified rabbit aorta constricting substance that had been described by Piper and Vane in 1969 [18] and was therefore presumed to be the same mediator. Since then, the vasoconstrictor effects of TXA_2 have been confirmed and it is now known that this lipid mediator also promotes vascular smooth muscle cell proliferation [7].

TXA_2 is sensed by the G-protein coupled TP receptor, which exists in two forms: TPα and TPβ. Both receptors are encoded by splice variants of a single gene and the translated isoforms differ in the structure of their carboxyl tails, [19]. Both of these isoforms couple to the G_q protein to signal the effects of TXA_2 [19].

PGE_2

Conversion of PGH_2 to PGE_2 is catalysed by four separate enzymes: micorosomal-PGE synthase-1 (mPGES-1), mPGES-2, cytosolic PGES (cPGES) and GSTμ [20]. Whilst mPGES-1 appears to couple functionally with COX-2, cPGES is reported to associate primarily with COX-1, providing a biochemical basis for a divergence of these isoforms in pathology and physiology [20]. In addition to this regulation of PGE_2 formation, the functions of this prostanoid are also regulated by the subtype of EP receptor expressed (EP_{1-4}) by the target tissue [21]. Within the cardiovascular system, EP_1 and EP_3 signal increases in vascular smooth muscle tone by increasing inositol triphosphate and decreasing adenylyl cyclase, respectively, and EP_3 increases platelet reactivity [21]. In contrast, EP_2 and EP_4 both signal increases in adenylyl cyclase activity, resulting in decreased vascular smooth muscle tone and platelet reactivity [21].

PGD_2

Isomerisation of PGH_2 to PGD_2 can be catalysed by either the lipocalin-type or haematopoietic PGD synthase (L-PGDS or H-PGDS) [22]. Similarly to PGE_2 and PGI_2, PGD_2 acts on platelet DP receptors to increase adenylyl cyclase and inhibit platelet aggregation [23].

1.2 Pharmacological inhibition of COX isoforms

The observation by Vane that a family of drugs known as non-steroidal anti-inflammatory drugs (NSAIDs) shared the common property of reducing prostaglandin production identified the inhibition of COX enzymes as the mechanism of action of this drug class [24]. NSAIDs are classified functionally as drugs that exhibit the common properties of being anti-inflammatory, analgesic and anti-pyretic and the identified compounds with these properties are structurally diverse [11, 25]. It is these structural differences that govern the biochemical interactions between the enzyme and inhibitor [11], leading to the varied pharmacology of drugs in this class [26].

NSAIDs are widely used in the treatment of pain, inflammation and swelling, forming the mainstay of treatment in patients with rheumatoid arthritis and osteoarthritis. As many as 25 million prescriptions are written for NSAIDs each year [27] and large quantities of these drugs are also sold over the counter [28].

The role of COX-2 as an inducible regulator of inflammation indicates that this isoform, and not COX-1, is the primary therapeutic target of NSAIDs [28]. However, the additional anti-thrombotic properties of aspirin (which are discussed later in this chapter) are believed to be overwhelmingly COX-1 dependent [29].

The inhibitory mechanisms of NSAIDs vary between chemical classes and aspirin is an irreversible, time-dependent inhibitor with differential effects on COX-1 and COX-2 [11]. By acetylating a specific serine molecule, Ser529 [30-32], aspirin provides steric hindrance of COX-1/arachidonic acid binding, preventing enzyme activity [11, 33]. In contrast, acetylation of the equivalent serine on COX-2 prevents the

generation of PGH_2 although the conversion of arachidonic acid to 15-(R)-hydroxyeicosatetraenoic acid is catalysed [11, 34].

1.2.1 Gastrointestinal effects of NSAIDs

A major limitation of NSAID therapy is their propensity to cause gastrointestinal (GI) toxicity, which was first reported in 1938 [35, 36]. NSAID use increases the basal risk of gastrointestinal events approximately 3-fold [37] and the occurrence of dyspepsia is sufficient to prompt discontinuation of NSAID therapy in up to 15% of rheumatoid arthritis patients [36, 38].

In an attempt to calculate the rate of death from adverse GI events attributable to NSAID use, Tramèr and colleagues evaluated studies performed before 1997, concluding that 1 in 1200 patients die from gastroduodenal complications caused by NSAID therapy [39]. More recent evaluation of the literature (up to 2008) has revealed that whilst mortality associated with GI bleeding and perforation of any cause has reduced over time (from 12% to 7%), major bleeding events associated with NSAID use have an approximately 20% risk of death, an increase from 15% in pre-1997 studies [40].

The mechanisms of NSAID-induced GI injury are multi-factorial although a reduction of gastric mucosal prostaglandins is believed to be an important factor [41]. The anti-platelet effect of some NSAIDs, including aspirin, can contribute to the injury by preventing effective wound healing at sites of erosions [36, 41]. In addition, some NSAIDs may also contribute to injury by direct topical irritation of the gastric mucosa [36, 41].

1.2.2 Isoform selective NSAIDs – a strategy to reduce GI risk

The suggestion that the therapeutic and adverse effects of NSAIDs may be attributable to inhibition of distinct COX isoforms, where COX-2 inhibition is advantageous and COX-1 inhibition is deleterious [25, 42, 43] led to the investigation of NSAID COX isoform selectivity, with the aim of identifying COX-2 selective compounds [25]. Further investigation of this hypothesis included comparison of *in vitro* COX selectivity with the occurrence of GI toxicity, showing that compounds more selective for COX-1 than for COX-2 are associated with a greater incidence of GI damage [25, 44].

The hypothesis that COX-1 inhibition is deleterious also drove the development of NSAIDs with a greater selectivity for COX-2, with the aim of sparing COX-1 and thus reducing GI injury [28]. Newly identified compounds with a greater selectivity for COX-2 were grouped as a subset of NSAIDs known collectively as 'coxibs' [28]. These drugs tend to be larger molecules that can exploit the structural differences in COX isoform active site structure. The substitution of isoleucine to valine at positions 434 and 523 in COX-2, relative to COX-1, results in a larger substrate channel [9]. The presence of a side-pocket on COX-2 accommodates these molecules and prevents arachidonic acid binding, whereas the reduced dimensions of the COX-1 active site prevent these molecules from binding to this isoform [28].

The first two 'coxibs' to gain approval for use in the United Kingdom were celecoxib and rofecoxib [28], and randomized clinical trials with both of these compounds indicated a reduction in gastrointestinal damage compared to traditional NSAIDs [45, 46]. The CLASS study reported that in a 12 month period patients taking only celecoxib had fewer symptomatic ulcers and ulcer complications than patients taking either diclofenac or ibuprofen [46]. Interestingly, this beneficial effect was abrogated in a small subset of patients who were also taking a low dose of aspirin (for

cardioprotection) in combination with either celecoxib or a traditional NSAID (diclofenac or ibuprofen) [46], consistent with other data reporting a significant risk of GI complications with very low doses of aspirin alone [47]. In fact, those taking aspirin were more likely to suffer GI complications regardless of their study drug randomization [46]. In the VIGOR study the GI toxicity of rofecoxib, a more selective inhibitor of COX-2 than celecoxib [44], was compared to naproxen, with all other NSAID use prohibited [45]. In this study, the relative risk of upper GI events was reduced by approximately 50% by using rofecoxib as opposed to naproxen, and the risk of severe complications was reduced by approximately 60% [45]. Whilst these data demonstrated a clear reduction in GI toxicity with this highly COX-2 selective NSAID randomization to rofecoxib in the VIGOR study carried a four-fold increase in risk of myocardial infarction, although the incidence of such events was low [45].

1.2.3 NSAIDs and cardiovascular risk

The apparent adverse cardiovascular events reported in the VIGOR study were originally explained as a function of naproxen-associated cardioprotection [45]. However, in a later placebo controlled trial investigating the chemopreventive effects of strong COX-2 inhibition the pro-thrombotic effects of rofecoxib were confirmed [48]. Rofecoxib appeared to double the risk of myocardial infarction and ischaemic stroke over a 3-year period when compared to placebo [48].

The biological basis for this increased risk is unclear, although hypotheses have been suggested [9, 49]. In general, it is believed that the relative levels of PGI_2 and TXA_2 are important in maintaining cardiovascular health, and in particular to avoid thrombotic events [9, 49]. This is largely based on the effects of low dose aspirin at inhibiting thrombotic events, an effect attributed to the reduction of platelet TXA_2 [29] and that dysfunctional IP receptors are associated with increased atherogenesis [21].

Healthy volunteer studies show that urinary PGI_2, but not TXA_2, metabolites are decreased with the use of COX-2 selective NSAIDs celecoxib [50] and rofecoxib [51], giving rise to the hypothesis that these drugs reduce prostacyclin by inhibiting endothelial COX-2 [49]. Whilst this explanation may seem appealing it is unfortunately not supported by evidence to show that COX-2 is present within the healthy vascular endothelium [9]. In healthy endothelial cells cultured in static conditions or under persistent shear stress for a period of up to seven days, COX-1 is the predominant isoform responsible for the production of PGI_2 [52]. Low doses of aspirin are able to inhibit excretion of the same urinary PGI_2 metabolite although the exact source of this product is not known [53].

Since the realisation of an association between cardiovascular risk and more COX-2 selective NSAIDs there has been much interest in determining whether or not this is a class effect of all NSAIDs. A large meta-analysis reported by Trelle et al. concluded that all of the NSAIDs studied were associated with an increased cardiovascular risk, although the nature of the risk, i.e. myocardial infarction, thrombotic stroke, varied between drugs [54]. The results of this meta-analysis demonstrate that COX-2 selectivity does not define the risk associated with a particular drug, as had been hypothesized based on observational data. However, as at therapeutic doses all these drugs inhibit COX-2, it is not possible to rule out loss of COX-2 function as the cause of this risk.

To understand how cyclo-oxygenases influence thrombotic risk it is important to consider the platelet and the mechanisms of vascular thrombosis.

1.3 Platelets

Platelets are enucleate, discoid cells formed from megakaryocytes. The primary physiological role of platelets is to arrest bleeding from damaged vessels, the mechanisms of which overlap with the pathophysiological role of platelets in arterial thrombosis.

1.3.1 Formation of platelets

Platelets are derived from megakaryocytes, precursor myeloid cells that reside primarily within the bone marrow [55]. The differentiation of megakaryocytes from haematopoietic stem cells is critically regulated by the cytokine thrombopoietin (TPO), which is essential for platelet formation [55, 56]. Treatment of cultured megakaryocytes with TPO *in vitro* has allowed direct visualization of platelet formation, suggesting that platelets form from elongated pseudopodia known as proplatelets [57]. Structures larger than platelets are known to be released into the circulation from megakaryocytes in bone marrow sinusoids [58] and recent work by Thon and colleagues proposes that these are proplatelets, which subsequently develop into mature platelets within the circulation [59]. The proplatelet fission model of platelet release suggests that proplatelets divide at cleavage furrows, leaving exposed ends from which platelets are released [59]. Although this process occurs over several days *in vitro*, the rate of platelet release increases with subsequent fission events, which are increased by shear forces, suggesting that it could occur much more rapidly *in vivo* [59].

1.3.2 Mature platelet structure

Mature platelets are approximately 2μM in diameter and are characterized by an extensive cytoskeleton, an open canalicular system and the presence of granules containing biologically active molecules [60].

Platelets contain three types of secretory granules: alpha granules, dense granules and lysosomes [61]. The most abundant granules are alpha granules, of which there is 50-80 per platelet [61]. These granules contain fibrinogen, von Willebrand's factor (vWF) and a large number of proteins that are either expressed on the platelet surface or released into the plasma [61]. Granule subpopulations with variable contents have been reported [62, 63], suggesting that granule formation can be regulated to allow responses tailored to specific stimuli [64]. Dense granules are less abundant than alpha granules (3-8 per platelet [65]) and contain low molecular compounds, such as ADP, ATP, calcium and serotonin, which are secreted to recruit other platelets, drive positive feedback mechanisms and possibly modify vascular function [65, 66]. Lysosomal granules secrete acid hydrolases that potentially breakdown platelet aggregates and may contribute to inflammatory responses [61, 67].

1.4 Platelets in vascular injury

In the normal arterial circulation, under laminar flow, platelets are marginalized to the luminal surface of the vessel – a result of their relatively small size in comparison to other blood cells [68]. Although the potential for platelet-vessel interactions is high under such conditions, platelets remain in a quiescent state, and are only activated at sites of vessel injury where sub-endothelial matrix components or other thrombogenic substrates are exposed [69]. The mechanisms of platelet activation, thrombus formation and the physiological inhibition of these processes are described in the following sections, initially in general terms and then in the specific context of arterial thrombosis in vascular disease.

1.4.1 Platelet adhesion

The first stage of arterial thrombus formation is platelet adhesion, the mechanism of which is determined by the conditions of flow, or more specifically shear, within the vessel [70, 71]. At shear rates between 1000-10000 s^{-1}, which reflects the shear rates experienced in large and small arteries [72], the initiation of platelet adhesion is dependent upon interactions between the A1 domain of immobilized vWF and the glycoprotein 1b (GPIb) [71, 73]. GPIb exists on the platelet surface in the GPIb/V/IX complex [74], whereas vWF that is present in either the extracellular matrix or plasma is immobilized onto the surface of collagen fibrils, or microfibrils, depending on the depth of vascular injury [70]. This early adhesion, known as platelet tethering, can also occur through the interaction of immobilized vWF and platelet integrin $\alpha_{IIb}\beta_3$ [70] and it has been suggested that 'outside-in' signaling through these receptors contributes to further platelet activation [71]. Platelets tethered at sites of vascular injury can interact with exposed collagen fibrils, which bind to the platelet collagen receptors GPVI and integrin $\alpha_2\beta_1$ [75]. The interaction of GPVI with collagen promotes platelet activation, including activation of integrin $\alpha_2\beta_1$, resulting in firm platelet adhesion and further activation [75].

1.4.2 Platelet activation

Platelet activation can be described as the second stage of thrombus formation and involves a wide range of platelet agonists, which act through specific G-protein coupled and glycoprotein receptors [69]. Platelet activation occurs in platelets that are adhered to the site of vascular injury and, through paracrine mediators, to freely circulating platelets in proximity to the injury [69]. The distinct mechanisms of different platelet activators are described in detail below.

Collagen

As briefly described above, exposed collagen fibrils can interact with the GPVI receptors present on the surface of platelets that have adhered at a site of vascular injury. GPVI is an immunoglobulin that activates platelets by phosphorylation of an immunoreceptor tyrosine-based activation motif (ITAM) on the Fc receptor (FcR) γ-chain [76, 77]. The FcR γ-chain dimer is co-expressed with GPVI on the surface of platelets [78] and the importance of this activation pathway is outlined by the lack of aggregation to collagen in FcR γ-chain deficient platelets [79]. ITAM phosphorylation triggers a cascade of downstream protein phosphorylation, initiated by the Src tyrosine kinases Lyn and Fyn [75]. This eventually leads to activation of phospholipase Cγ2 (PLCγ2) and generation of the second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), which increase intracellular calcium and activate protein kinase C, respectively [80]. The rise in intracellular calcium causes granule secretion, releasing ADP, and stimulates TXA_2 formation, and these secondary mediators lead to further platelet activation by their actions on G-protein coupled receptors [81]. Protein kinase C activation by GPVI is involved in the switching of integrin $\alpha_2\beta_1$ to a high affinity conformation [82], which stabilizes the adhesion of platelets to collagen, reinforcing GPVI signaling [81, 83].

Differentiation of collagen receptor responses can be achieved *in vitro* with the use of collagen-related peptides, such as CRP-XL [75, 84]. These short peptides contain a repeated GPO motif, which activates the GPVI receptor but not the integrin $\alpha_2\beta_1$ [76, 84, 85].

Thrombin

The cleavage of prothrombin to thrombin is regulated by tissue factor, a cofactor for the activation of coagulation factor X [86]. Activated factor X (Xa) and factor Va co-

operate in the cleavage of prothrombin to thrombin [86], which takes place on the surface of platelets [87]. Tissue factor is normally segregated from the blood although the two come into contact at sites of vascular injury [88]. Thrombin activates platelets through the protease-activated receptors (PARs), of which, PAR1 and PAR4 are found on human platelets [86]. PARs, which are coupled to G_q and $G_{12/13}$ [87], are activated by N-terminus cleavage of the receptor, exposing a sequence that autoligates the body of the receptor and effects intracellular signaling [86]. The activation sequence of PAR1 is SFLLRN, which is synthesised as thrombin receptor activating peptide (TRAP-6) and used for *in vitro* investigation of PAR1 activation [89].

G_q-linked PAR signaling leads to the activation of phospholipase Cβ (PLCβ) and the downstream generation of the second messengers IP_3 and diacylglycerol (DAG) [86, 87]. IP_3-mediated increases in extracellular calcium promote the activation of cytosolic phospholipases, leading to TXA_2 production [86, 87]. In addition, PAR activation of G_q is associated with granule secretion and integrin $\alpha_{IIb}\beta_3$ activation [90].

ADP

ADP is stored in dense granules (see above) and released from platelets upon activation by other agonists, including collagen, thrombin and TXA_2 [69]. ADP acts on two G-protein coupled receptors, $P2Y_1$ and $P2Y_{12}$, with differential effects determined by associated G-protein type. $P2Y_1$ couples with G_q to mediate ADP-induced shape change and initiates weak, reversible aggregation through PLCβ, whereas $P2Y_{12}$ couples with G_i to amplify and sustain platelet aggregation [91]. The effects of $P2Y_{12}/G_i$ activation are to inhibit the activation of adenylyl cyclase, reducing intracellular cAMP [87, 92], and to upregulate phosphoinositide 3-kinase activation [93]. In addition, G_i signaling initiated by either ADP or adrenaline is required for

activation of the small GTPase Rap1b [94, 95], which is a critical regulator of integrin $\alpha_{IIb}\beta_3$ activation [96].

Adrenaline

Platelets express the α_{2A}-adrenergic receptor coupled to the G_i-type G-protein G_z [97]. Agonism of this pathway by adrenaline is only a weak stimulus of platelet activation, although G_z signaling can potentiate the effects of other platelet agonists [87].

TXA_2

TXA_2 release after platelet stimulation is a consequence of *de novo* synthesis, possible once arachidonic acid has been liberated from the cell membrane by cytosolic phospholipases A_2 [80]. The TXA_2 receptor (TP receptor) is coupled to G_q and $G_{12/13}$ in human platelets [98, 99]. TP receptor activation promotes granule secretion and ADP release, which appears to be one of the primary mechanisms of TXA_2-induced platelet activation [100, 101]. TP receptor activation can be investigated *in vitro* using the TXA_2-mimetic U46619.

1.4.3 Platelet aggregation

Platelet aggregation is the incorporation of activated platelets into a growing thrombus, and this process occurs concurrently with platelet activation by the mechanisms described above [69, 80]. Stable thrombus formation relies upon the activation of the fibrinogen receptor, integrin $\alpha_{IIb}\beta_3$, without which the aggregation process will reverse [69]. Integrin $\alpha_{IIb}\beta_3$ activation results in a change of conformation, revealing a RGD-binding sequence, allowing interaction of the integrin with fibrinogen, vWF and fibronectin [102]. Activation occurs by ‘inside-out’ signaling

and is mediated by the GTPase Rap1b in response to both G_i and G_q signaling [95, 103]. The cross-linking of $\alpha_{IIb}\beta_3$ on adjacent platelets, by fibrinogen, results in the formation of a platelet aggregate [102].

1.4.4 Inhibitors of platelet activation and aggregation

PGI_2

The formation and extra-platelet actions of PGI_2 are described above. The actions of PGI_2 on the platelet are mediated through the G-protein coupled IP receptor [104], which associates with the G_s protein [105]. G_s signaling leads to activation of adenylyl cyclase and an increase in intracellular cAMP [87], opposing some of the effects of $P2Y_{12}$ receptor activation, and therefore the secondary effects of TXA_2 [106]. Recently, Cattaneo and Lecchi reported that PGI_2 may contribute to the anti-platelet effect of $P2Y_{12}$ receptor blockade by increasing intracellular cAMP [107] (see below).

The short half-life of PGI_2 (<3 minutes) under physiological conditions [14] means that this factor is no longer active in platelet samples used for *in vitro* analysis of platelet activation.

Nitric oxide

Nitric oxide (NO) is a potent inhibitor of platelet aggregation [70], generated by the actions of endothelial nitric oxide synthase and this gaseous mediator diffuses from the vessel wall to act on platelets [108]. Within the platelet NO activates soluble guanylyl cyclase, which produces the second messenger cyclic guanosine monophosphate (cGMP) [108]. At least some of the antiplatelet effects of NO appear to be mediated through cGMP kinase I (cGKI), as loss of this enzyme results in increased platelet adhesion and aggregation [109]. Specific functions of cGKI include

the phosphorylation of Rap1b (decreasing its function) [110] and the inhibition of G_q and G_i protein signaling, potentially counteracting the effects of ADP and TXA_2 [108].

Adenosine

Adenosine is generated from ADP by the ecto-ADPase CD39, which is expressed on the surface of endothelial cells [80]. Ligation of the platelet α_{2A}-adrenergic receptor by adenosine activates adenylyl cyclase, leading to upregulation of the second messenger cAMP [87]. Recent work by Iyú et al. has shown that this pathway can demonstrate enhanced antiplatelet effects in the presence of a of $P2Y_{12}$ antagonist [111], an important observation as the reversible $P2Y_{12}$ receptor blocker ticagrelor has the potential to increase circulating adenosine [112].

1.5 Arterial thrombosis in vascular disease

The role of platelets of primary interest to this thesis is that of atherothrombosis [113, 114], where stable platelet aggregates form at the site of atherosclerotic plaque rupture [114].

Atherosclerosis is a chronic, progressive, inflammatory disorder of the arterial system that begins with endothelial dysfunction and progresses to the development of lipid-rich lesions within the vasculature, and platelets are involved in each stage of the disease [115]. The process is initiated by high circulating levels of low-density lipoproteins, free radicals and other factors which can cause vascular inflammation [115]. At sites of inflammation adherent platelets release a number of cytokines, chemokines and growth factors, in addition to the mediators discussed above, promoting endothelial dysfunction and leukocyte recruitment [113]. The pro-inflammatory milieu created supports leukocyte transmigration, the differentiation of monocytes and macrophages to foam cells, the recruitment and activation of T-cells

and smooth muscle cell migration to create early plaque lesions, or fatty streaks [113, 115, 116]. As plaque development progresses, lesions form a fibrous cap and a lipid-rich necrotic core [115]. By this stage, plaques are also rich in collagen and tissue factor and are therefore highly thrombogenic [116]. As plaque progression continues the fibrous cap may thin, resulting in disintegration or rupture and contact between the thrombogenic contents and circulating platelets [117]. This leads to platelet activation, aggregation and thrombus formation, by the mechanisms described previously, and can present clinically as acute coronary syndrome, myocardial infarction or ischaemic stroke [114]. If not treated promptly these conditions are associated with a high risk of morbidity and mortality [114].

Acute coronary syndrome (ACS) describes three distinct ischaemic states: unstable angina, non-ST segment elevation myocardial infarction (nSTEMI) and ST segment myocardial infarction (STEMI) [118]. Although STEMI can be diagnosed by electrocardiography, unstable angina and nSTEMI can be hard to distinguish on the basis of electrical activity, although myocardial infarction can be detected by the presence of cardiac necrotic markers such as troponin-I and –T [118]. ACS represents a spectrum of disease both clinically and pathologically [119], with unstable angina and nSTEMI typically associated with non-occlusive thrombi formed predominantly of platelets [120] whereas STEMI is associated with the inclusion of red cells and greater activation of the blood coagulation cascade [121].

In addition to providing a thrombogenic surface atherosclerotic lesions can affect platelet activation and thrombosis by altering the flow dynamics within the vessel [71]. Arterial stenosis, which can occur in atherosclerosis, increases flow velocity and therefore increases shear rates in affected areas of the vasculature in excess of 10000 s^{-1} [122]. This creates a shear-gradient, which Nesbitt and colleagues demonstrate is sufficient to activate platelets and promote thrombus formation even

in the absence of soluble mediators [123]. In this environment, aggregates form in the low shear region immediately downstream of the stenosis, where deceleration supports turbulent flow [123].

1.5 Pharmacological prevention of thrombotic vascular events

A number of anti-platelet drugs are currently available for use, or under investigation, as therapies to prevent the occurrence, or recurrence of thrombotic vascular events such as myocardial infarction and stroke.

1.5.1 Aspirin

As mentioned earlier in this chapter, aspirin irreversibly inhibits COX isoforms by acetylating the Ser529 residue in the active site of the enzyme, preventing the formation of prostanoids from arachidonic acid. In the platelet this reduces the production of TXA_2 and inhibits platelet aggregation [29]. Aspirin is orally active and dependent on the formulation used has an oral bioavailability of up to 50%, with peak plasma levels achieved within 40 minutes of ingestion [29]. Hepatic and gastric mucosal hydrolysis of aspirin to salicylic acid limits the half-life of aspirin in plasma to only 15-20 minutes, although as platelets encounter concentrated aspirin levels in the hepatic portal circulation, and the effects of aspirin are irreversible, this is sufficient to provide a lasting drug effect with once daily dosing in patients with normal platelet turnover [29]. In diabetics, who have an increased rate of platelet turnover, once daily aspirin dosing has a limited efficacy and it may be that more frequent doses are required to provide a full anti-thrombotic effect [124].

Recurrent meta-analyses performed by the Antithrombotic Trialists' Collaboration have shown that aspirin is also effective at reducing the rate of serious vascular events – including myocardial infarction, ischaemic stroke and vascular death – by

almost 25% in those who have previously had an ischaemic vascular event, or are at a particularly high risk of arterial thrombosis [125, 126]. The most recent meta-analysis by this consortium confirmed that higher doses of aspirin (500-1500mg daily) are no more effective than low dose aspirin (75-100mg) in preventing serious vascular events [125].

For the primary prevention of serious vascular events aspirin reduces the risk of serious vascular events by 12% and this effect is predominantly due to an approximately 20% reduction in the risk of myocardial infarction [127]. However, aspirin is currently only indicated in those with identifiable cardiovascular disease as in the absence of overt cardiovascular disease the associated increased risk of haemorrhagic stroke and extracranial bleeding probably outweighs the thrombotic benefit [127].

1.5.2 $P2Y_{12}$ receptor blockers

Clopidogrel

Clopidogrel, a member of the thienopyridine class of drugs, is an orally administered pro-drug, metabolized in vivo to generate an active metabolite that inhibits the ADP $P2Y_{12}$ receptor [128]. The clopidogrel active metabolite irreversibly inhibits the $P2Y_{12}$ receptor for the life of the platelet thereby inhibiting ADP-induced amplification of platelet aggregation and thrombus formation [128].

Clopidogrel metabolism was traditionally believed to be cytochrome P450 (CYP450) mediated although recent work by Bouman et al. suggests a role of the Paraoxonase-1 enzyme in generating the clopidogrel active metabolite [129]. Whichever mechanism of hepatic metabolism predominates, the bioavailability of active metabolite, and therefore the anti-platelet effect of this drug, is highly variable

between individuals [130, 131]. On average, clopidogrel administration results in a 50% reduction in ex vivo ADP-induced platelet aggregation [130, 131].

Clinically, clopidogrel can be used as an alternative to aspirin [132, 133], although this is uncommon and primarily clopidogrel use is reserved as an adjunct to aspirin in dual anti-platelet therapy in those at particularly high risk of recurrent ischaemic events [133].

Prasugrel

Another member of the thienopyridine class, prasugrel is similar to clopidogrel in that it is a pro-drug that relies upon hepatic metabolism to generate its active metabolite although the pharmacokinetic and pharmacodynamic profiles of these two drugs differ [134]. Maximum plasma concentrations of prasugrel active metabolite are detected 30 minutes after a single 60mg oral dose [134] and, after four hours, the inhibition of ADP-induced platelet aggregation is consistently inhibited by approximately 80% in healthy volunteers [128]. In the same subjects, clopidogrel had a varied effect against the same platelet stimulus and the extent of platelet inhibition correlated with active metabolite generation [128].

In the clinic, prasugrel is superior to clopidogrel when used as a dual anti-platelet therapy with aspirin for the prevention of vascular events in acute coronary syndrome managed with percutaneous coronary interventions [135]. The increased anti-platelet effect of prasugrel in pre-clinical and clinical use does, however, lead to an increase in bleeding [135, 136].

Ticagrelor

Ticagrelor is the first orally active, reversible inhibitor of the $P2Y_{12}$ receptor and belongs to the cyclopentyl-triazolo-pyrimidine class of drugs [137]. Although ticagrelor is metabolized to AR-C124910XX both of these compounds have equivalent efficacy and a long half life (~6-9 hours) in the steady state after twice daily dosing (bid) [137]. 100mg bid ticagrelor in healthy volunteers has a strong pharmacodynamic effect, as assessed by platelet aggregation in response to 20μM ADP, with >90% inhibition of this response sustained for 24 hours after 5 days dosing [137]. A similar dose was used in the DISPERSE and DISPERSE-2 studies of response to ticagrelor, with aspirin, in patients with stable atherosclerosis [138] or non-ST elevation acute coronary syndrome [139], respectively. DISPERSE showed that in this population with stable cardiovascular disease the peak plasma concentration of ticagrelor was reached in approximately 3 hours [138]. After 1 day of 100mg bid ticagrelor, the inhibition of ADP-induced platelet aggregation was very high (~80%) and this was sustained in the steady state after 28 days [138]. DISPERSE-2 compared a 90mg bid dose of ticagrelor to 75mg clopidogrel (both with aspirin) in patients undergoing standard management of non-ST elevation acute coronary syndromes, assessing the primary end-point of major and minor bleeding rates over 4 weeks [139]. At 4 weeks, the rate of minor, but not major bleeding was elevated in those randomized to ticagrelor, compared to clopidogrel. Patients in the 90mg bid ticagrelor group were also more likely to suffer from dyspnoea and/or nausea than those taking clopidogrel [139].

In the PLATO trial – a large, multi-center double-blind randomized controlled trial to compare the efficacy of ticagrelor and clopidogrel (both with aspirin) to reduce the composite end-point of myocardial infarction, ischaemic stroke and vascular death after acute coronary syndrome events – ticagrelor was associated with a lower rate in the primary end-point than clopidogrel [140].

1.5.3 Dual anti-platelet therapy of aspirin and a $P2Y_{12}$ receptor blocker

The rationale of dual anti-platelet therapy is that by inhibiting independent pathways of platelet activation, drugs can be used in combination to achieve either additive or synergistic effects. Typically, dual anti-platelet therapy consists of aspirin and a $P2Y_{12}$ receptor blocker, on the assumption that aspirin inhibits TXA_2 formation and $P2Y_{12}$ inhibition prevents ADP-dependent aggregation, although as explained above, TXA_2-dependent platelet aggregation is largely mediated by ADP release and so these inhibitors have some overlapping effects.

The hypothesis that aspirin is additive to clopidogrel in the prevention of secondary thrombotic events has not been fully tested as most trials have investigated the effect of aspirin alone or in addition to clopidogrel, without the inclusion of a clopidogrel only group [141]. Neither has this hypothesis been tested in the use of dual anti-platelet therapy for primary prevention in those at high risk of thrombotic vascular events, although the CHARISMA trial showed that the use of aspirin and clopidogrel in primary prevention is no more effective than aspirin alone, and is not recommended [142]. That clopidogrel is additive to aspirin across a general cardiovascular population is, however, now well established [133], and the newer drugs prasugrel and ticagrelor are both more effective than clopidogrel when used with aspirin for secondary prevention [135, 140].

Hypothesis

Considering the multiple effects of inhibiting $P2Y_{12}$-dependent pathways of platelet activation, and the complex association between pharmacological inhibition of cyclo-oxygenases and the risk of thrombotic vascular events, this thesis aims to investigate the hypothesis that modulation of COX function within the cardiothoracic system may have differential effects upon pathways of platelet activation.

Aims

Investigations were focused on the differential effects of aspirin when used with $P2Y_{12}$ receptor blockers *in vitro* and *in vivo* in order to determine the role of aspirin with more potent $P2Y_{12}$ inhibitors. Different assays of platelet activation were used in order to understand the experimental parameters that affect the apparent pharmacology of these drugs. In addition, experiments in genetically modified mice were included to investigate the role of different COX isoforms in the cardiothoracic system.

This approach was structured into the following specific aims:

1. To investigate *in vitro* the interaction between cyclo-oxygenase and $P2Y_{12}$ pathways as targets for anti-thrombotic therapy.
2. To conduct a small clinical study investigating the anti-platelet and extra-platelet effects of aspirin in the presence of strong $P2Y_{12}$ receptor blockade in healthy volunteers.
3. To use genetically modified mice and elucidate the relative roles of COX-1 and COX-2 in the production of prostanoids in cardiothoracic tissues under normal and acute inflammatory conditions.

Chapter 2: Materials and Methods

Materials

Acetylsalicylic acid (aspirin)	Sigma, UK
Adenosine Diphosphate	Labmedics, UK
Adrenaline	Labmedics, UK
Ascorbic Acid	Sigma, UK
Calcium Ionophore A23187	Sigma, UK
Chlorhexadine	Sigma, UK
CHRONO-LUME reagent	Labmedics, UK
Creatine Phosphokinase	Sigma, UK
Diclofenac	Sigma, UK
Dimethyl Sulfoxide	VWR, UK
Ethanol	VWR, UK
Heparin	CP Pharmaceuticals Ltd, UK
Horm Collagen	Nycomed, Switzerland
Lepirudin (Refludan®)	Celgene, UK
Lipopolysaccharide	Sigma, UK
N-propyl gallate	Sigma, UK
Phenylmethanesulfonyl Fluoride	Sigma, UK
Phosphate Buffered Saline	Sigma, UK
Phosphocreatine	Sigma, UK
Prasugrel Active Metabolite	AstraZeneca, Sweden
Ticagrelor	AstraZeneca, Sweden
TRAP-6 amide	Bachem, UK
Tri-sodium citrate	Sigma, UK
Trizma Base	Sigma, UK
U46619	Cayman Chemical Company, USA

Methods

Below are detailed the common experimental and analytical methods used in multiple chapters of this thesis. Experimental or analytical methods specific to a single chapter are described in that chapter, as detailed in the table of contents.

2.1 Ethics and consent

All experiments using human volunteers were approved by the St Thomas's Hospital Research Ethics Committee (Ref. 07/Q0702/24). All volunteers gave informed written consent to participate in the studies.

2.2 Screening of healthy blood donors

After providing written consent (see section 2.1), potential blood donors were screened based on answers to a structured questionnaire, previous and current medical history, current medication and physical examination (temperature, pulse rate, heart rhythm and blood pressure) to determine health status. All donors were aged 18-40 and both male and female donors were included in all studies unless otherwise stated.

2.3 Venepuncture

Venepuncture was performed in the ante-cubital fossa, using a 19 gauge (Fr) butterfly needle. The blood was drawn into a syringe containing either 3.2% tri-sodium citrate or 250µg/ml lepirudin, as specified in each chapter. In either case, anticoagulant and blood were in a 1:9 ratio at the end of venepuncture to give a final concentration of 0.109M sodium citrate or 25µg/ml lepirudin.

2.4 Preparation of PRP and PPP from whole blood

Platelet-rich plasma was obtained by the centrifugation of whole blood at 175 x g for 15 minutes, after which point the PRP fraction was removed, taking care not to extract the buffy coat. Platelet poor plasma was obtained by the centrifugation of PRP or whole blood at 1300 x g for 2 minutes.

2.5 Treatment of PRP with anti-platelet drugs

Aspirin was prepared to a concentration of 1M in 100% ethanol and then diluted in PBS to a concentration 10-200 times the required final concentration before addition at 1:10 or 1:200 to PRP. PAM and ticagrelor were prepared to a concentration of 10mM in DMSO and then diluted to a concentration 100-200 times the required final concentration before being added 1:100 or 1:200 into PRP. After addition of anti-platelet agents, PRP was incubated in a water bath at 37ºC for 30 minutes before use in platelet assays.

2.6 Preparation of platelet agonists

ADP, TRAP-6 and U46619 were all prepared from 1mM stock diluted in PBS. Horm collagen 1mg/ml was diluted in an isotonic glucose buffer (supplied with this agonist). Lyophilized ristocetin was reconstituted to 30mg/ml and further diluted in dH_2O. Arachidonic acid (100mM stock in 100% ethanol) and adrenaline (1mM stock) were both diluted into 0.1% ascorbic acid in PBS. All agonists were prepared at a concentration 10 times that required and added 1:10 to PRP in platelet assays.

2.7 96-well plate light transmission aggregometry

To assess the aggregation of platelets in 96-well plates a modified light transmission

method was used, as previously described [143]. 100µl samples of PRP were placed into the individual wells of a 96-well microtitre plate containing 10µl agonist (at 10x final concentration). On the top row of the plate samples of PRP and PPP were included as controls. The plate was then placed into a Tecan Sunrise 96-well plate reader, pre-warmed to 37°C, and absorbance of 595nm light was measured every 15 seconds for 16 minutes (64 measurements), with lateral shaking at 738 rpm for 7 seconds between readings. Percentage aggregation was calculated with reference to the absorbance of PPP as a surrogate for 100% aggregation. A typical plate layout is shown in figure 2.1.

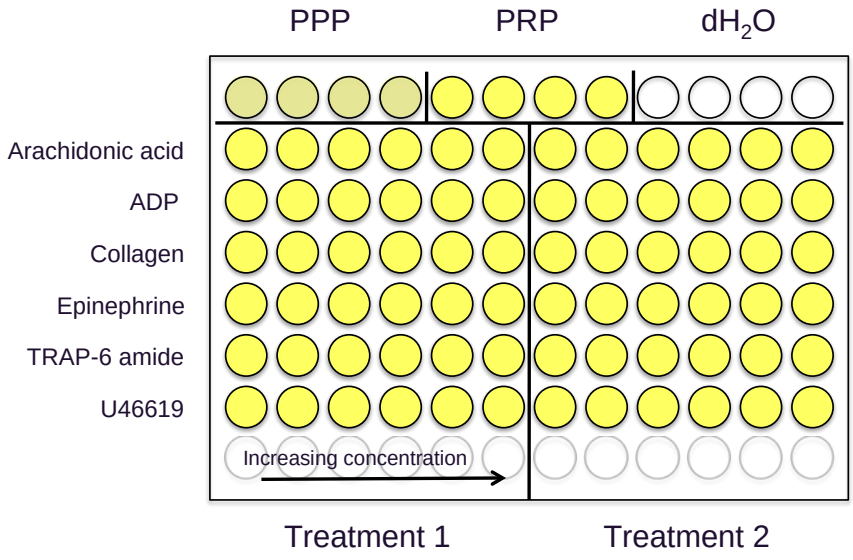


Figure 2.1 Typical layout of 96-well plate aggregometry to six agonists

2.8 Thromboxane B_2 assays

At the end of aggregation, COX activity was halted by the addition of diclofenac, to a

final concentration of 1mM, to limit further TXA_2 generation. Samples were then centrifuged and the plasma supernatant removed and stored. The stable metabolite TXB_2 was assayed as a measure of TXA_2 formation in the supernatant using either of two different commercially available enzyme-linked immunoassay kits or a Homogeneous Time-Resolved Fluorescence (HTRF) assay. Each method is described below:

2.8.1 TXB_2 short incubation assay

This competitive immunoassay was performed in accordance with the package insert (Catalog no. 901-002; Assay Designs, Ann Arbor, MI, USA). Samples were diluted 1:10 in diluent and assayed parallel to known TXB_2 standards and a maximum binding control on a 96-well microtitre plate, coated with goat anti-rabbit IgG. After the addition of anti-TXB_2 rabbit polyclonal antibody and alkaline phosphatase conjugated TXB_2 the plate was incubated at approximately 500rpm, on an orbital shaker, for 2 hours. The contents of each well were aspirated, and the plate washed 3 times. After washing, the plate was developed with p-nitrophenyl phosphate solution and the absorbance of each well determined at 405nm. From the absorbance data, the percentage binding of known standards was calculated in reference to the maximum binding (zero TXB_2) control wells, plotted against the logarithm of concentration and analysed by non-linear regression using a four-parameter logistical fit model. Unknown samples were expressed in a similar fashion, interpolated from this standard curve and corrected for dilution.

2.8.2 TXB_2 overnight incubation assay

This competitive EIA was performed in accordance with the package insert. Samples were diluted 1:10 in diluent and assayed parallel to known TXB_2 standards and a maximum binding control on a 96-well microtitre plate, coated with goat anti-IgG

antibodies. The plate was incubated for 18 hours after the addition of a TXB_2-acetylcholinesterase conjugate and TXB_2-specific rabbit antiserum. After washing, Elmann's reagent solution was added, the plate was covered and allowed to develop for 45-60 minutes on an orbital shaking platform. Once developed, the absorbance of each well was determined at 405nm. From the absorbance data, the percentage binding of known standards was calculated in reference to the maximum binding (zero TXB_2) control wells, plotted against the logarithm of concentration and analysed by non-linear regression using a four-parameter logistical fit model. Unknown samples were expressed in a similar fashion, interpolated from this standard curve and corrected for dilution.

2.8.3 TXB_2 HTRF assay

In this competitive immunoassay, supernatant TXB_2 and d2-labeled TXB_2 compete for binding to a specific anti-TXB_2 antibody labeled with cryptate. When cryptate is excited in close proximity to d2, i.e. after binding of d2-TXB_2 and cryptate-anti-TXB_2, it donates energy to sustain the emission of fluorescence from d2, detectable at 665nm. Sustained d2 fluorescence is not detected in the absence of a donor fluorophore. Fluorescence from excited cryptate is detectable at 620nm. To compensate for variations in signal transmission between samples, comparisons are made between the ratio of emission at 665nm/620nm.

Before the assay was run, samples were diluted in assay diluent (50mM phosphate buffer, pH7.0 containing 0.2% BSA). TXB_2 standards were similarly prepared by dilution in assay diluent. 10µl diluted supernatant or standard was then added to wells of a 384-well black microtitre plate by our in-house automated pippetor, Robbie (CAS1200, Corbett Robotics, Australia). 5µl TXB_2-d2 conjugate and 5µl anti-TXB_2 cryptate conjugate were added to each well and the plate incubated at room

temperature for 1hr. A positive (10µl diluent, 5µl d2 conjugate, 5 µl cryptate conjugate) and negative control (10µl diluent, 5µl buffer, 5µl cryptate) was included on each assay plate. After excitation at 320nm, emission at 665nm and 620nm was determined for each well. The mean 665/620nm ratio of each sample/control was then calculated from individual replicates and used to calculate the Delta $F_{\%}$ for each sample/standard, where Delta $F_{\%}$ = $((Ratio_{sample}-Ratio_{neg\ control})/Ratio_{neg\ control})*100$.

Delta $F_{\%}$ values of known standards were plotted against the logarithm of concentration and analysed by non-linear regression using a four-parameter logistical fit model. Unknown samples were interpolated from this standard curve and corrected for dilution.

2.9 Data analysis

Instrument specific outputs were manipulated in Microsoft Excel. All data analysis and graphing was performed in GraphPad Prism v5 (GraphPad Software Inc, CA, USA).

2.10 Statistical methods

The statistical methods used are described in each chapter.

Chapter 3: *In vitro* platelet activation in the presence of aspirin and prasugrel active metabolite

3.1 Introduction

As mentioned previously, aspirin is the first choice of anti-platelet therapy in those at risk of thrombotic cardiovascular events. The rationale of aspirin use is that inhibition of platelet cyclo-oxygenase by aspirin reduces TXA_2 production, and therefore TXA_2-driven platelet aggregation. The co-prescription of a $P2Y_{12}$ receptor blocker such as clopidogrel or prasugrel is common during periods of increased thrombotic risk (i.e. after coronary stent implantation) and this additional therapy aims to inhibit ADP-driven platelet aggregation. However, *in vitro* data suggest that $P2Y_{12}$ receptor blockade alone can inhibit TXA_2-driven platelet aggregation in two ways, firstly by reducing the production of TXA_2 in response to platelet agonists in platelet rich plasma [144] and secondly, by inhibiting platelet aggregation in response to TP receptor stimulation in washed platelets [100, 101]. This suggests a possible redundancy of aspirin in the presence of effective $P2Y_{12}$ receptor blockade [141].

In this chapter, I have aimed to investigate the interaction between cyclo-oxygenase and ADP-$P2Y_{12}$ pathways of platelet activation, using aspirin and prasugrel-active metabolite (PAM) as pharmacological tools. Specifically, I aimed to identify the contribution of aspirin to the inhibition of platelet activation and aggregation in the presence of strong $P2Y_{12}$ receptor blockade by PAM.

3.2 Methods

3.2.1 Preparation of platelet solutions

PRP and PPP were prepared from whole blood anti-coagulated with either citrate or lepirudin, as described in sections 2.3 and 2.4.

3.2.2 Incubation of platelet-rich plasma

100 µl samples of PRP were pre-incubated for 30 min at 37°C with PAM (0.1-10µM), and/or aspirin (30 µM), or vehicle (0.1% DMSO) before being transferred to a 96-well microtitre plate for determination of agonist-induced platelet aggregation.

3.2.3 Platelet aggregation

Platelet aggregation was determined by 96-well plate light transmission aggregometry, as described in section 2.7. The plate format was adapted to assess either, antagonist and agonist both at multiple concentrations, or a fixed concentration of antagonist against multiple agonist concentrations.

3.2.4 ATP-ADP release

To determine the release of ATP and ATP+ADP from the platelets, a modified luminometric method was used [14, 15]. 100 µl of PRP was placed in each well of a white clear bottomed 96-well plate containing 10 µl Horm collagen (0.1-30µg/ml), TRAP6 amide (0.1-30µM), or U46619 (0.1-30µM) and placed in the plate reader as for platelet aggregation above. After five minutes, the plate was removed from the plate reader. To measure ATP release CHRONO-LUME reagent (1:5 v/v) was then added to individual wells; while to measure ATP+ADP release 50 µl of substrate containing phosphocreatine (1.5mM), creatine phosphokinase (3000 U/ml) together with CHRONO-LUME reagent (1:5 v/v) was added to wells. The plate was shaken at

350 rpm for two minutes at 37°C and then luminescence determined on a 1450 Microbeta Trilux (Perkin Elmer, Waltham, MA, USA). The luminescence of a 20 nM ATP standard in PPP was used to calculate the concentrations of ATP and ATP+ADP released.

3.2.5 Determination of cyclo-oxygenase inhibition

To determine the direct effects of aspirin and PAM upon platelet cyclo-oxygenase activity, 100 µl samples of PRP were pre-incubated for 30 min at 37°C with PAM (0.1-10µM), aspirin (30µM), or vehicle (0.1% DMSO) before being placed into the individual wells of a 96-well microtitre plate and then rapidly frozen, to lyse the platelets. Plates were then rapidly thawed and incubated (37°C) for 16min with shaking in the presence of arachidonic acid (1mM). At the end of this period the 96-well plates were processed for the assessment of TXA_2 production in the same way as for 96-well plates containing intact platelets.

3.2.6 TXB_2 assay

At the end of the PRP aggregation monitoring (i.e. 16min), cyclo-oxygenase activity was halted by the addition of 1mM diclofenac, the samples were centrifuged at 1300 x g for 10 minutes at 5°C, and the supernatants removed and frozen. TXB_2 was determined using the short assay kit, as described in section 2.8.1.

3.2.7 Statistical analysis

All statistical analyses were conducted using GraphPad Prism v5 (GraphPad Software Inc, CA, USA). Inhibitory concentration-response curves were plotted and analysed by the four parameter logistic equation: Y=Bottom + (Top-Bottom)/(1+10^((LogIC50-X)*HillSlope)). Whereas agonist concentration-response

curves were plotted and analysed according to a similar equation: Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))

Concentration-response curves were compared by two-way ANOVA and Bonferroni post-tests. In each case, $p \leq 0.05$ was taken as a significant change.

3.3 Results

To assess the role of cyclo-oxygenase inhibition by aspirin in the presence of strong $P2Y_{12}$ receptor blockade it was necessary to perform concentration-ranging experiments with PAM and then select an appropriate concentration to study platelet aggregation and secondary mediator release in response to a range of platelet agonists. Aspirin 30μM has previously been established as an effective concentration in the aggregation assay used here [145].

3.3.1 Concentration-ranging of prasugrel-AM

As shown in figure 3.1, platelet aggregation to ADP (0.1-30μM) was inhibited in a concentration-dependent manner by PAM. At 1μM, PAM inhibited aggregation responses between 50-75% although at 3μM platelet aggregation in response to all concentrations of ADP was inhibited by at least 95%. The inhibitory effect of PAM against U46619-induced platelet aggregation was similar to that seen for ADP [figure 3.2], with a strong effect of 3μM PAM against multiple concentrations of U46619. In contrast, the inhibition of collagen-induced aggregation varied with concentration of collagen - PAM had a stronger effect against lower (0.1-3μg/ml) concentrations of collagen, and a weaker effect at high concentrations (10-30μg/ml) [figure 3.2].

Based on these observations, 3μM PAM was used in further experiments to assess the effects of aspirin and PAM on secondary mediator release and platelet aggregation.

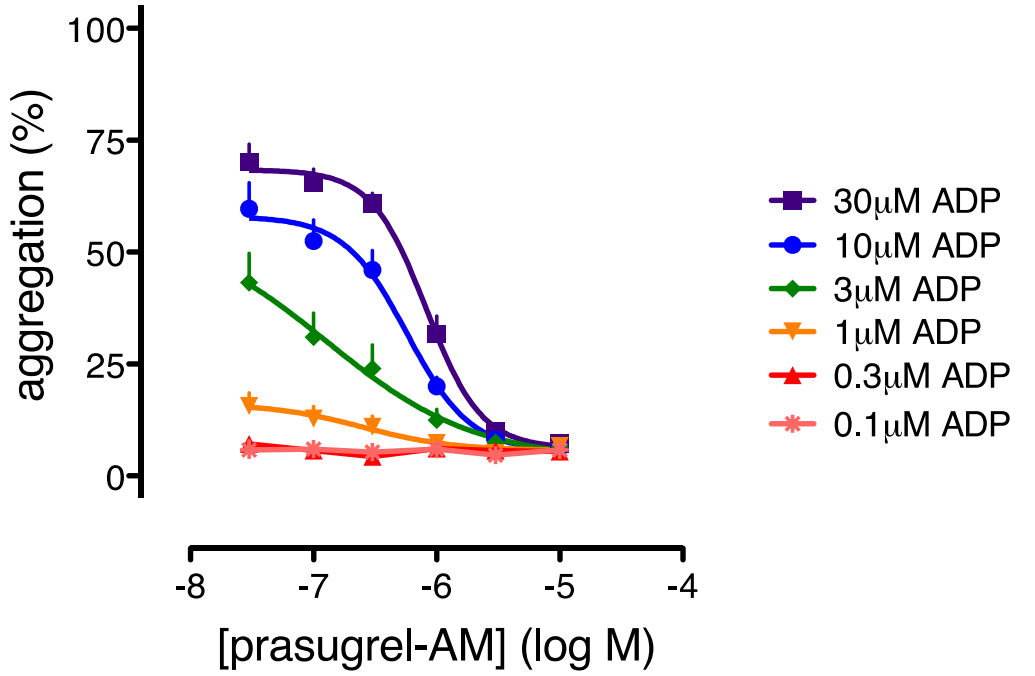


Figure 3.1 The effect of prasugrel active metabolite on ADP-induced platelet aggregation.

Concentration dependent inhibition of 0.1-30μM ADP induced platelet aggregation by PAM (0.03-10μM). Data represent the mean ± s.e.m. of responses from 6 individuals.

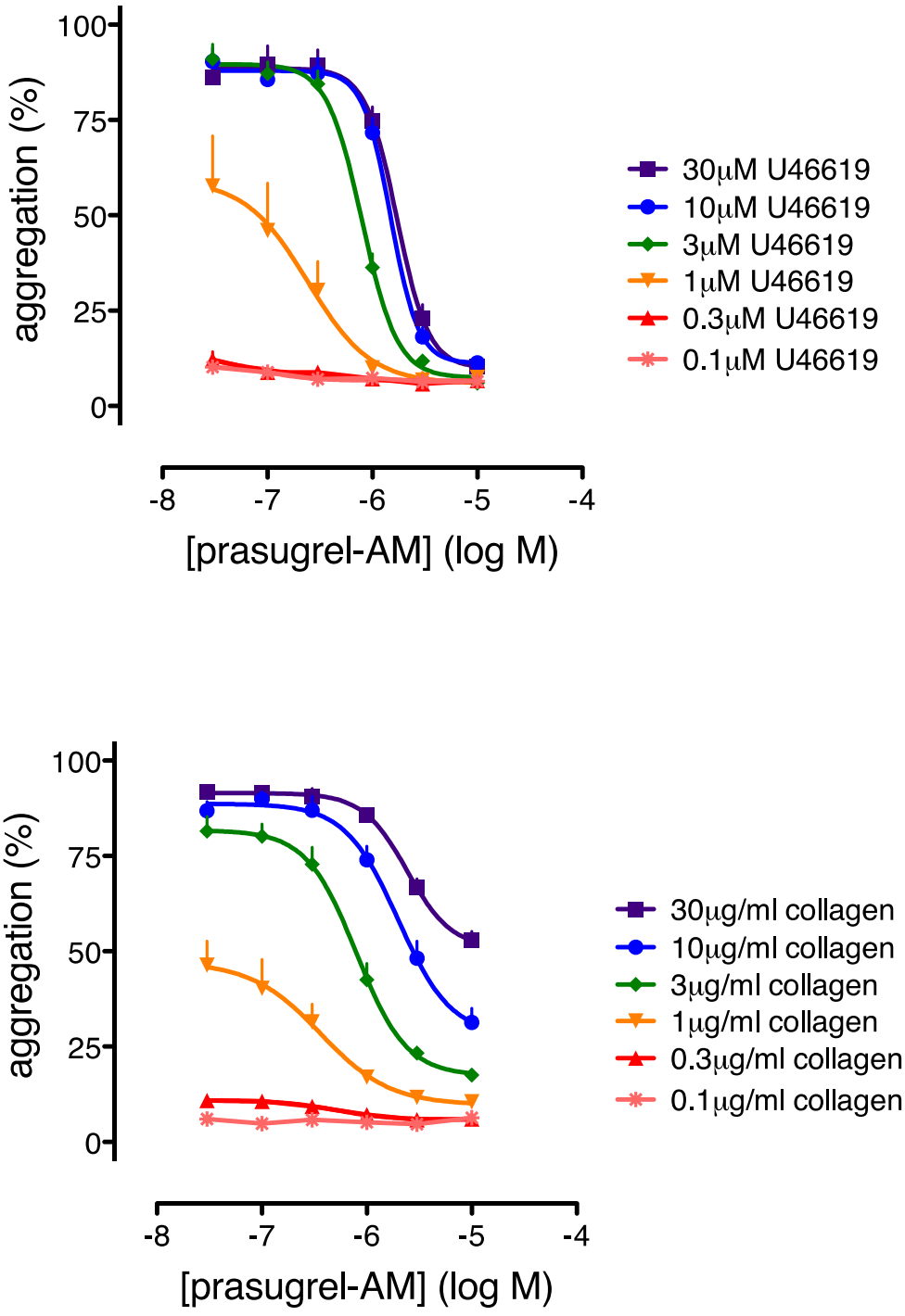


Figure 3.2 The effect of prasugrel active metabolite on U46619 and collagen induced platelet aggregation.

Platelet aggregation in citrated PRP induced by 0.1-30μM U46619 (upper panel) and 0.1-30μg/ml collagen (lower panel) is inhibited in a concentration dependent manner by PAM. Data represent the mean ± s.e.m. of responses from 6 individuals.

3.3.2 The individual and combined effects of aspirin and PAM on platelet aggregation in citrated PRP

The effects of aspirin 30µM and PAM 3µM, individually and in combination, on platelet aggregation induced by arachidonic acid, ADP, collagen, adrenaline, TRAP-6 amide, U46619 and ristocetin were assessed, and are shown in figure 3.3. Ristocetin was included to demonstrate that vWF-dependent agglutination was present. Aspirin alone inhibited aggregation to arachidonic acid, collagen and adrenaline, although it had no effect on ADP, TRAP-6 amide or U46619 induced-aggregation, whereas PAM alone inhibited the aggregation induced by all agonists to a similar, or greater, extent to the inhibition by aspirin. When aspirin and PAM were used in combination the inhibition achieved by PAM alone was not furthered for any agonist, with the exception of collagen.

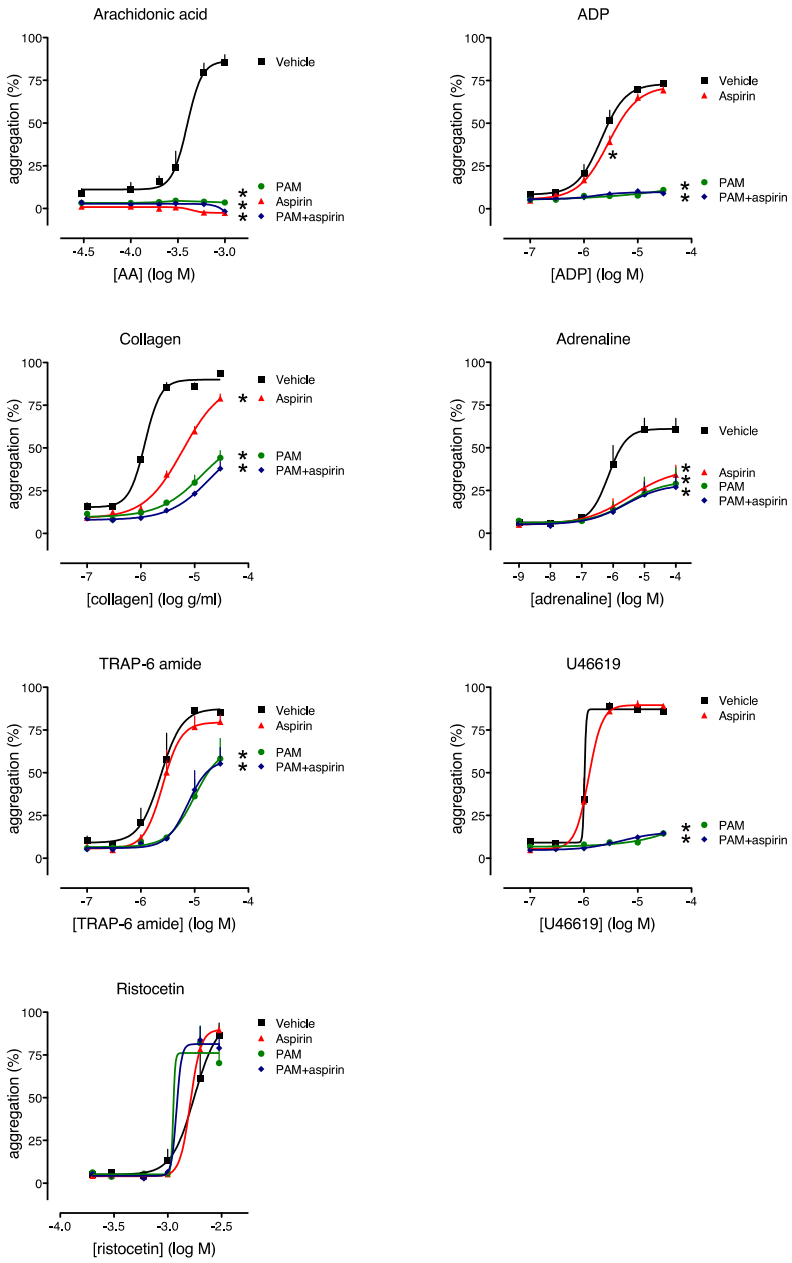


Figure 3.3 The effect of PAM and aspirin, alone and in combination, against platelet aggregation induced by six different agonists (citrated PRP).

Platelet aggregation in citrated PRP induced by arachidonic acid (0.03-1mM), ADP (0.1-30µM), collagen (0.1-30µg/ml), adrenaline (0.001-100µM), TRAP-6 amide (0.1-30µM), U46619 (0.1-30µM) and ristocetin (0.02-3mM) in the presence of PAM 3µM and/or aspirin 30µM. Data represent the mean ± s.e.m. of responses from 4 individuals. * indicates $p<0.05$ by two-way ANOVA compared to vehicle; † indicates $p<0.05$ in PAM+aspirin compared to PAM. Symbols at the end of lines indicate difference for all concentrations; symbols at points indicate a difference at that concentration.

3.3.3 The individual and combined effects of aspirin and PAM on TXA_2 production in citrated PRP

TXA_2 production in response to arachidonic acid, collagen and adrenaline was reduced by PAM alone, whereas aspirin alone, or in combination with PAM, abolished TXA_2 production in response to the same agonists [figure 3.4].

To determine if the reduction of TXA_2 production by PAM was due to a direct effect on platelet COX, TXA_2 production was assessed in pre-treated broken platelet preparations stimulated with arachidonic acid (1mM). Arachidonic acid stimulation resulted in considerable TXA_2 production in control samples (435±17 ng/ml TXB_2; mean±s.e.m., n=24), although this was markedly inhibited by aspirin (10, 30 and 100µM caused inhibitions of 46±11%, 79±4% and 90±3%, respectively; mean±s.e.m., n=4 for each; $p<0.05$). In contrast to its inhibitory effect on TXA_2 production during aggregometry studies PAM had no effect on broken platelets (1, 3 and 10µM caused inhibitions of 2±8%, 16±5% and 4±11%, respectively; mean±s.e.m., n=4 for each; n.s.) [figure 3.5].

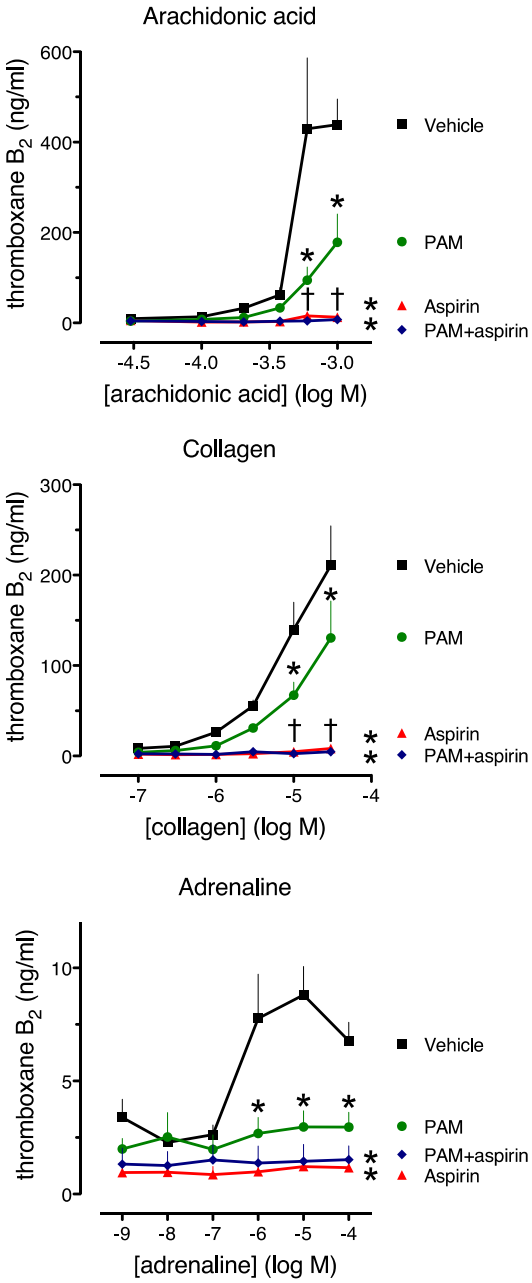


Figure 3.4 The agonist induced TXB_2 production by platelets treated with PAM and aspirin, alone and in combination (citrated PRP).

TXB_2 production in citrated PRP after platelet aggregation induced by arachidonic acid (0.03-1mM), collagen (0.1-30µg/ml) and adrenaline (0.001-100µM) in the presence of PAM 3µM and/or aspirin 30µM. Data represent the mean ± s.e.m. of responses from 4 individuals. * indicates $p<0.05$ by two-way ANOVA compared to vehicle; † indicates $p<0.05$ in PAM+aspirin compared to PAM. Symbols at the end of lines indicate difference for all concentrations; symbols at points indicate a difference at that concentration.

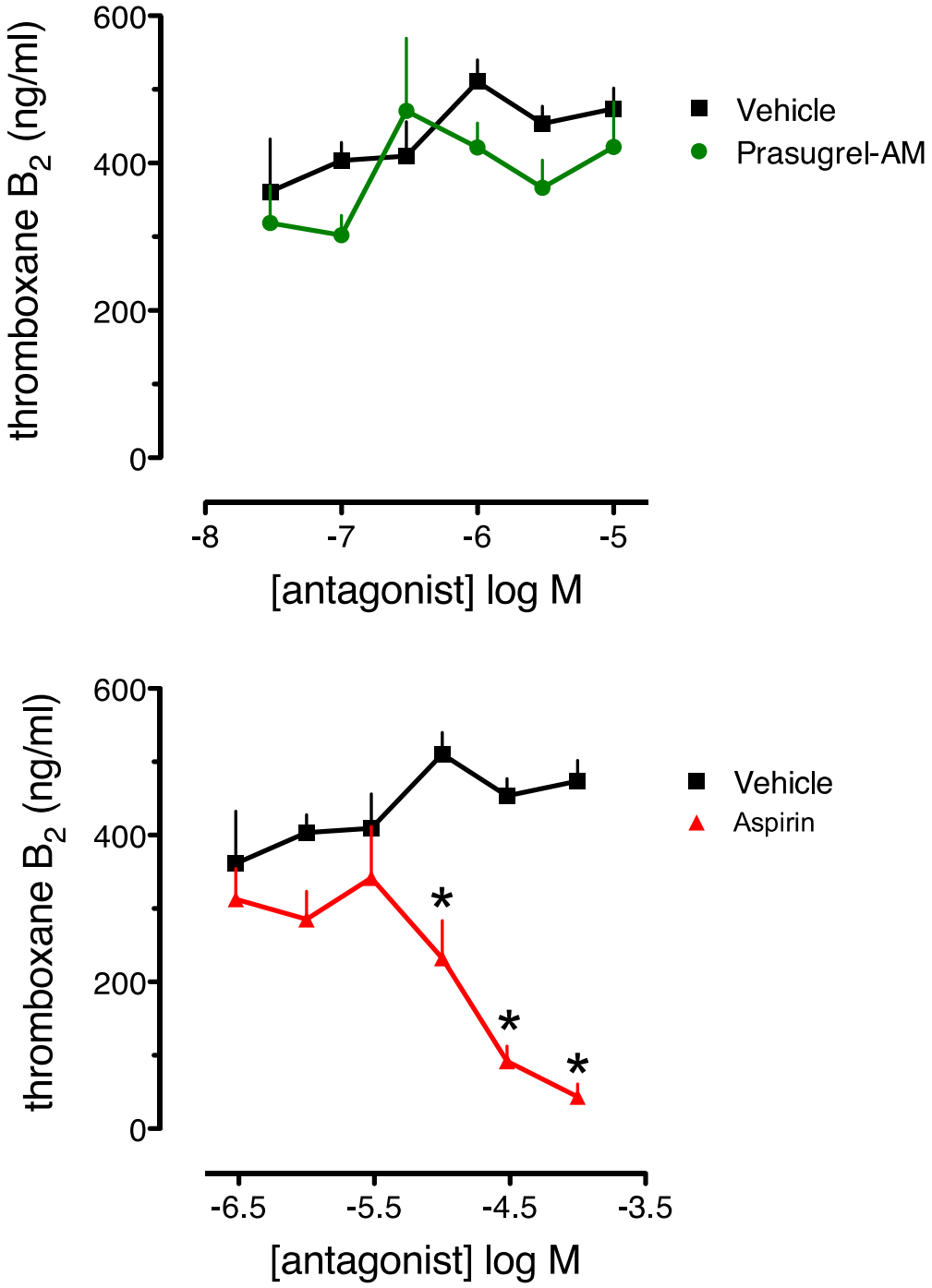


Figure 3.5 The inhibition of TXB_2 production by PAM is not a direct effect of cyclo-oxygenase inhibition.

TXB_2 production in platelets treated with PAM (0.03-10μM), aspirin (0.3-100μM) or vehicle (0.1% DMSO), lysed by freeze-thaw and then stimulated with arachidonic acid (1mM). Data represent the mean ± s.e.m. of responses from 4 individuals. * indicates $p<0.001$ by two-way ANOVA.

3.3.4 The individual and combined effects of aspirin and PAM on ATP and ATP+ADP release in citrated PRP

PAM and aspirin alone inhibited ATP and ATP+ADP release by collagen stimulated platelets and, consistent with the results observed for platelet aggregation, there was a small additional effect when PAM and aspirin were used in combination. Aspirin, however, had no effect on either TRAP-6 amide or U46619 induced ATP or ATP+ADP release. PAM did inhibit the nucleotide release induced by these agonists, although this was not furthered by the addition of aspirin. In each case, the effects of aspirin and PAM were similar on both ATP and ATP+ADP release [figure 3.6].

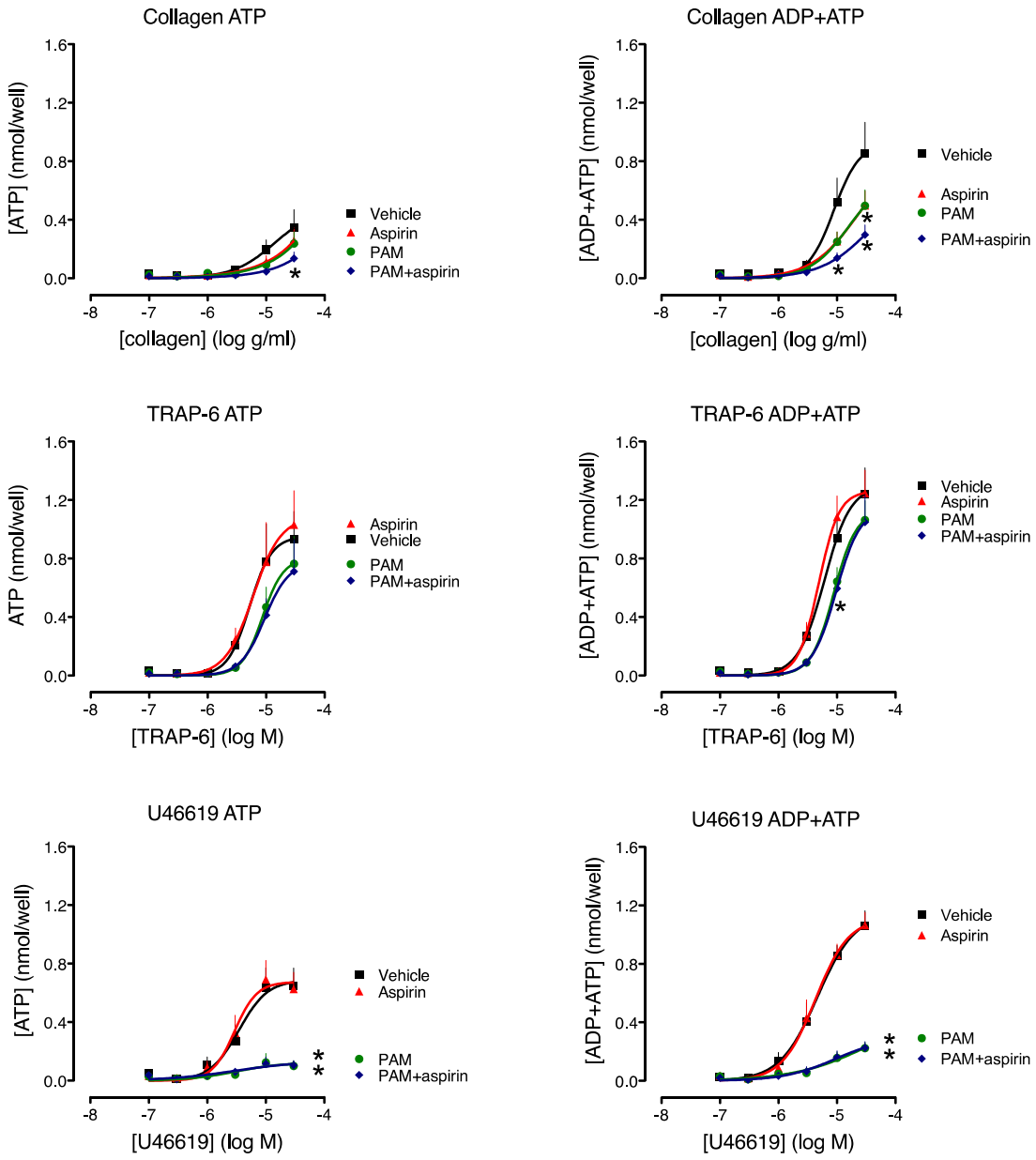


Figure 3.6 Agonist induced ATP and ADP release by platelets treated with PAM and aspirin alone and in combination.

ATP and ADP+ATP release from platelets in citrated PRP induced by collagen (0.1-30µg/ml), TRAP-6 amide (0.1-30µM) and U46619 (0.1-30µM) in the presence of PAM 3µM and/or aspirin 30µM. Data represent the mean ± s.e.m. of responses from 5 individuals. * indicates p<0.0.5 by two-way ANOVA compared to vehicle. Symbols at the end of lines indicate difference for all concentrations; symbols at points indicate a difference at that concentration.

3.3.5 The individual and combined effects of aspirin and PAM on platelet aggregation in lepirudinized PRP

To determine if the observations reported in sections 3.3.2 and 3.3.4 were influenced by the calcium chelating effects of tri-sodium citrate these experiments were repeated in PRP prepared from blood drawn into lepirudin. In vehicle treated samples the platelet aggregation in lepirudin was largely similar to that seen in citrate, although platelets in lepirudin appear to be slightly more sensitive to collagen, TRAP-6 amide and U46619 [figure 3.7]. The inhibition by aspirin and PAM also differed in comparison to citrated samples, although similarly, aspirin was no more effective than PAM against aggregation induced by any agonist, and in no instance did aspirin increase the anti-aggregatory effect achieved by PAM alone, except to a small extent in the case of collagen [figure 3.7].

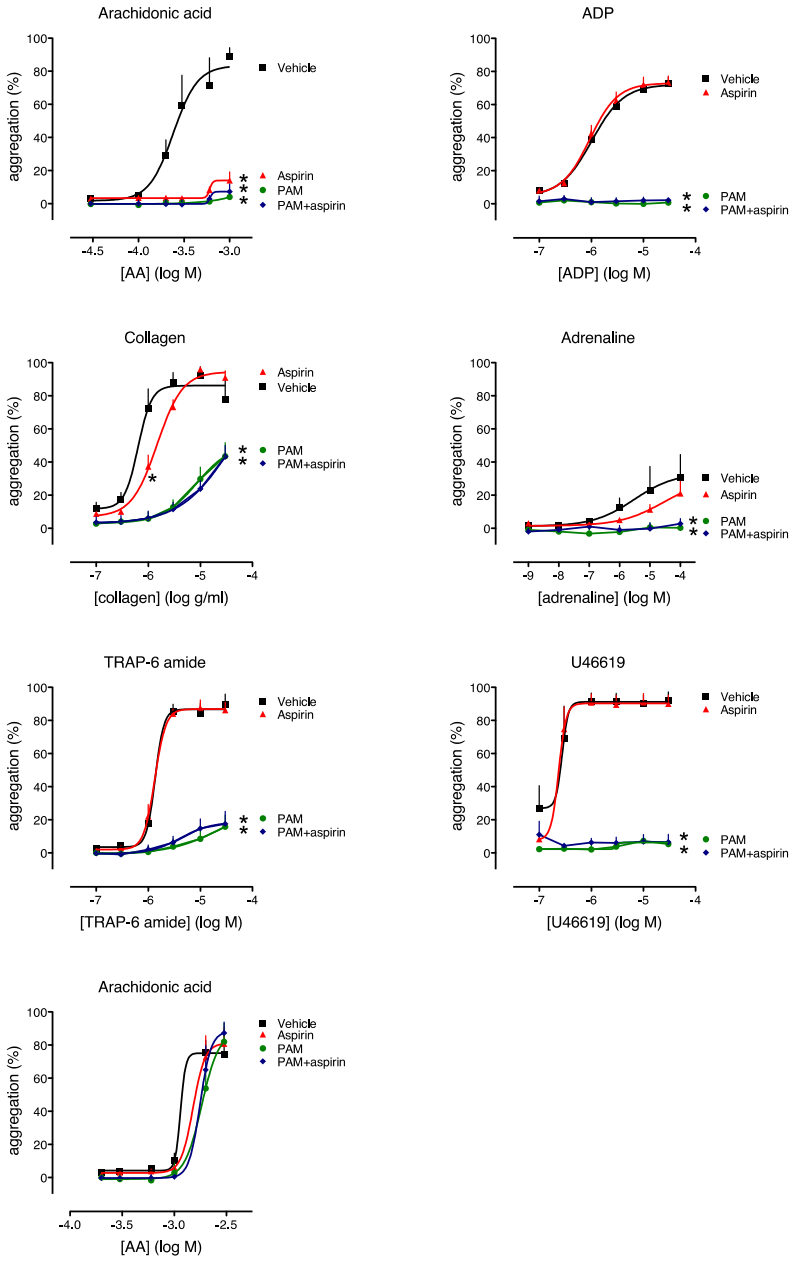


Figure 3.7 The effect of PAM and aspirin, alone and in combination, against platelet aggregation induced by six different agonists (lepirudinised PRP).

Platelet aggregation in lepirudinised PRP induced by arachidonic acid (0.03-1mM), ADP (0.1-30µM), collagen (0.1-30µg/ml), adrenaline (0.001-100µM), TRAP-6 amide (0.1-30µM), U46619 (0.1-30µM) and ristocetin (0.02-3mM) in the presence of PAM 3µM and/or aspirin 30µM. Data represent the mean ± s.e.m. of responses from 4 individuals. * indicates $p<0.0.5$ by two-way ANOVA compared to vehicle; † indicates $p<0.05$ in PAM+aspirin compared to PAM. Symbols at the end of lines indicate difference for all concentrations; symbols at points indicate a difference at that concentration.

3.3.6 The individual and combined effects of aspirin and PAM on TXA_2 production in lepirudinized PRP

TXA_2 production in lepirudinized PRP during platelet aggregation to arachidonic acid, collagen was approximately half that in citrate [figures 3.4 and 3.8. PAM partially reduced TXA_2 production, with an effect similar to that seen in citrate, and once again aspirin alone completely abolished TXA_2 production and abolished any remaining TXA_2 production when combined with PAM. TXA_2 production was not detectable in samples treated with adrenaline (0.001-100µM), despite some aggregation that appeared to be reduced in the presence of aspirin [figure 3.7].

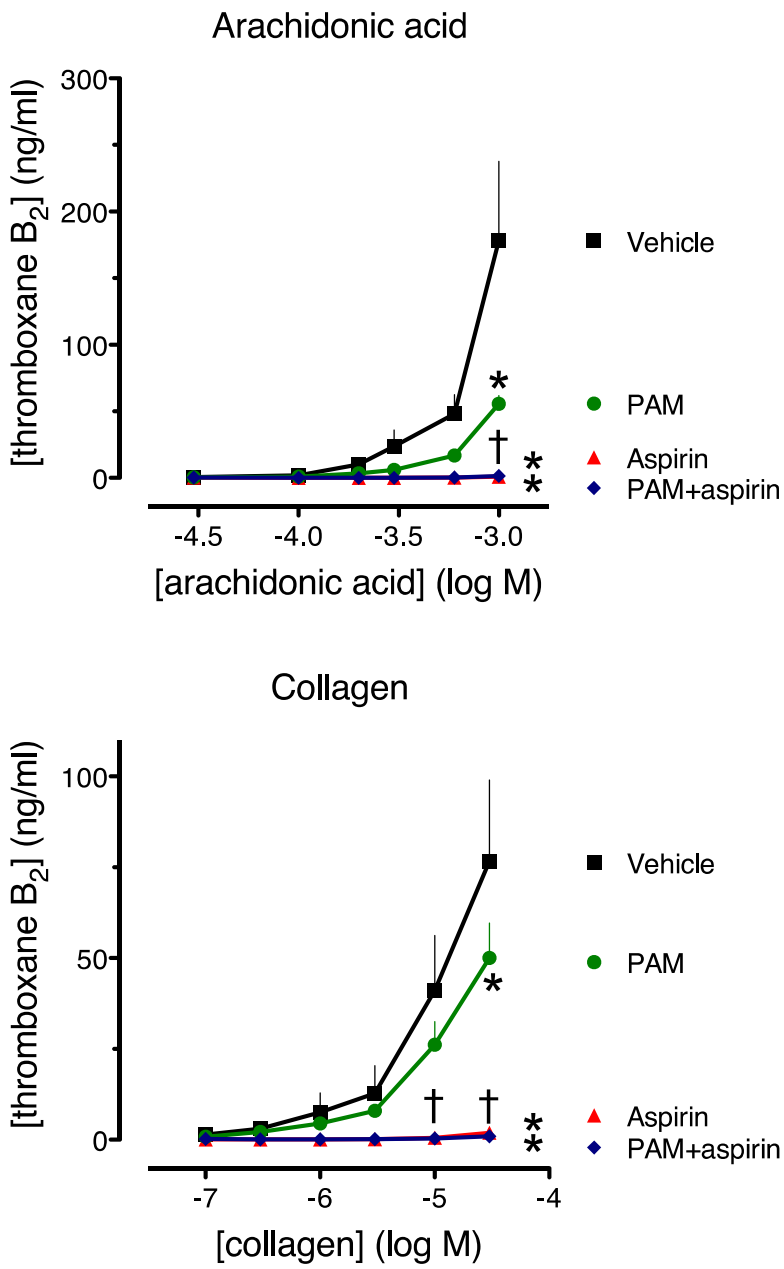


Figure 3.8 Agonist induced TXB_2 production by platelets treated with PAM and aspirin alone and in combination (lepirudinised PRP).

TXB_2 production in lepirudinised PRP after platelet aggregation induced by arachidonic acid (0.03-1mM), and collagen (0.1-30µg/ml) in the presence of PAM 3µM and/or aspirin 30µM. Data represent the mean ± s.e.m. of responses from 4 individuals. * indicates $p<0.05$ by two-way ANOVA compared to vehicle; † indicates $p<0.05$ in PAM+aspirin compared to PAM. Symbols at the end of lines indicate difference for all concentrations; symbols at points indicate a difference at that concentration.

3.4 Summary

30μM of aspirin was a sufficiently high concentration to completely inhibit agonist-induced platelet TXA_2 production, consistent with the clinical use of 75mg aspirin [145] and the concentration-ranging experiments performed indicated that 3μM of PAM, which is very close to the peak plasma concentration achieved with clinical use of prasugrel [134], provides strong $P2Y_{12}$ receptor inhibition. These concentrations were used to model *in vitro* the effects of aspirin in the presence of strong $P2Y_{12}$ receptor inhibition, with platelet activation and aggregation assessed using 96-well plate methods. The results reported here further confirm that these are sensitive assays to detect the inhibition of both COX and $P2Y_{12}$-dependent pathways of platelet activation.

Where platelet aggregation was reduced by aspirin alone (arachidonic acid, collagen, adrenaline) this was associated with the abolition of platelet TXA_2 production, although $P2Y_{12}$ receptor blockade alone achieved either an equal, or greater reduction of platelet aggregation with only partial inhibition of TXA_2 production. This is consistent with the inhibitory effect of PAM on ADP-$P2Y_{12}$ receptor signaling, blocking the actions of secondary mediator release. Furthermore, PAM fully inhibited dense granule secretion and platelet aggregation in response to TP receptor stimulation by U46619. The inhibition of TP receptor responses by $P2Y_{12}$ receptor blockade by this method is similar to that seen in washed platelets assessed by traditional LTA [100, 101], although others have reported a more limited affect of $P2Y_{12}$ receptor blockade against TP-specific responses in PRP [146, 147].

The inhibition of platelet aggregation in the presence of PAM was not furthered by the addition of aspirin, except to a small extent in the case of collagen-induced aggregation. Any effects of aspirin in this *in vitro* system can be considered

synonymous with the inhibition of TXA_2 production. As platelet TXA_2 production is increased by a low extra-cellular calcium concentration [148-151], as occurs with calcium chelation by sodium citrate, and to ensure that these results reflected the effects of PAM and aspirin in as close to a physiological environment as possible, platelet aggregation and TXA_2 production were also assessed in lepirudin. As expected TXA_2 production was decreased, although the trends for the effects on platelet aggregation of aspirin and PAM were maintained; platelet aggregation was still inhibited more by PAM than by aspirin, and aspirin had no additional effect, except to a small extent in the case of collagen.

The *in vitro* model used here is limited in that it assesses the contribution of aspirin in the presence of $P2Y_{12}$ receptor blockade for only a single concentration of both aspirin and PAM. In the next chapter, I will assess the contribution of varying concentrations of aspirin to both complete and partial $P2Y_{12}$ receptor blockade.

Whilst the concentrations of aspirin and PAM used here reflect relevant concentrations or biological effects and provide a useful insight they are not an adequate substitute for *in vivo* experimentation to determine the contribution of aspirin to platelet aggregation in the presence of prasugrel. This has been investigated in a small clinical study in Chapter 5.

The moderately strong inhibition of collagen-induced platelet aggregation by PAM reported in this chapter differs from reports assessing the utility of $P2Y_{12}$ receptor blockers in models of platelet aggregation using continuous, often high speed, stirring. In these cases, collagen-induced platelet aggregation was only weakly affected by $P2Y_{12}$ receptor blockade, although strongly by aspirin. Clearly the differences in sensitivity to these important pharmacotherapies should be resolved in order to improve the understanding of *ex vivo* platelet function testing, and this issue

has been addressed in Chapter 6.

Chapter 4: The additional effects of aspirin in the presence of partial and complete $P2Y_{12}$ receptor blockade

4.1 Introduction

In the previous chapter I examined the effect of aspirin in an *in vitro* model of platelet aggregation in the presence of strong $P2Y_{12}$ receptor blockade by PAM. However, whilst it may be possible to achieve such a high level of $P2Y_{12}$ receptor blockade by taking oral anti-platelet therapies such as prasugrel, it is plausible that at times the level of inhibition will be lower. For instance, clopidogrel, the most commonly used $P2Y_{12}$ receptor blocker is well known to have variable effects in mixed populations [152]. Clopidogrel is a pro-drug that relies upon two CYP450 metabolic steps for the generation of its active metabolite [153]. The prasugrel active metabolite is generated from prasugrel in only one CYP450-dependent step, improving the relative bioavailability of this drug [134]. The cyclo-pentyl-triazolo-pyrimidine ticagrelor is a novel, orally active $P2Y_{12}$ receptor blocker, which has been shown to have a greater therapeutic benefit than clopidogrel in Phase III testing [140]. In contrast to the thienopyridines, which are irreversible antagonists, the actions of ticagrelor are reversible. With both reversible and irreversible antagonists, effective platelet inhibition requires adherence to the prescribed dosing regimen, although in reality doses are missed both accidently and deliberately.

For these reasons I have conducted *in vitro* studies to determine the effects of aspirin in the presence of varying levels of $P2Y_{12}$ receptor blockade by the thienopyridine derivative, prasugrel-active metabolite, and the cyclo-pentyl-triazolo-pyrimidine, ticagrelor.

4.2 Methods

4.2.1 Preparation of platelet solutions

PRP and PPP were prepared from whole blood anti-coagulated with citrate, as described in sections 2.3 and 2.4.

4.2.2 Incubation of platelet-rich plasma

100 µl samples of PRP were pre-incubated for 30 min at 37°C with PAM (0.1-10µM) or ticagrelor (0.03-3µM), and/or aspirin (30 µM), or vehicle (0.1% DMSO) before being transferred to a 96-well microtitre plate for determination of agonist-induced platelet aggregation.

4.2.3 Platelet aggregation

Platelet aggregation was determined by 96-well plate light transmission aggregometry, as described in section 2.7. The plate format was adapted to assess either, an antagonist and an agonist both at multiple concentrations, or a fixed concentration of antagonist against multiple agonist concentrations.

4.2.4 TXB_2 assay

At the end of the PRP aggregation monitoring (i.e. 16min), cyclo-oxygenase activity was halted by the addition of 1mM diclofenac, the samples were centrifuged at 1300 x g for 10 minutes at 5°C, and the supernatants removed and frozen. TXB_2 was determined using the short assay kit, as described in section 2.8.1.

4.2.5 Statistical analysis

The analyses conducted in this chapter were as for chapter 3 [section 3.2.7].

4.3 Results

4.3.1 Concentration-ranging of ticagrelor, PAM and aspirin against ADP-induced aggregation

The effects of aspirin, PAM and ticagrelor against the aggregatory effects of a range of ADP concentrations were determined by antagonist concentration-ranging experiments. Whilst even high concentrations of aspirin had no appreciable effect on ADP-induced aggregation, both $P2Y_{12}$ receptor blockers caused concentration-dependent inhibition of aggregation [figure 4.1], although their potencies varied. Against 20µM ADP, ticagrelor was almost one order of magnitude more potent than PAM, as determined by log IC_{50} values for the inhibition of aggregation (ticagrelor, -6.463; PAM, -5.644). Whilst PAM was equipotent against all ADP concentrations, ticagrelor was yet more potent against aggregation induced by lower concentrations of ADP (log IC_{50} values: 10µM, -6.637; 5µM, -6.801; 2.5µM, -7.048; 1.25µM, -7.444).

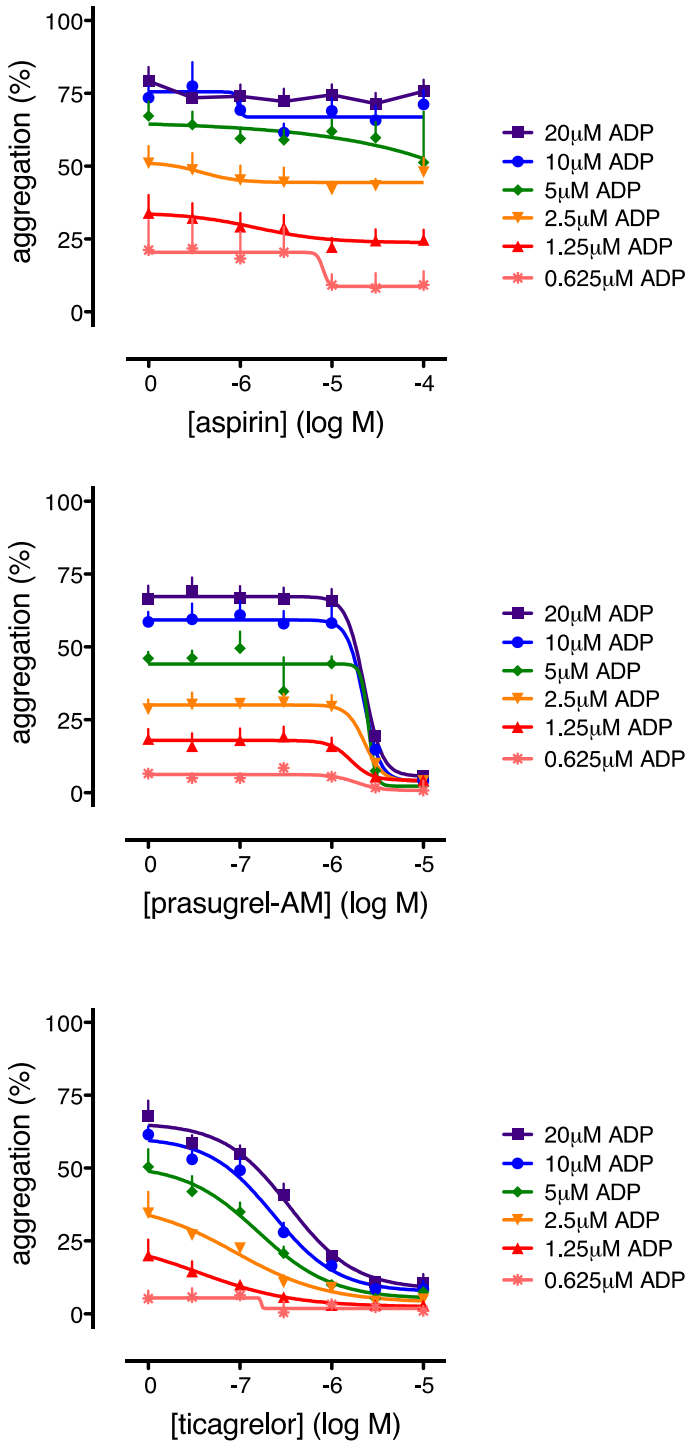


Figure 4.1 The effect of ticagrelor, PAM and aspirin on ADP induced platelet aggregation.

Concentration dependent inhibition of ADP (0.625-20μM) induced platelet aggregation by aspirin (0.1-100μM), PAM (0.03-10μM) and ticagrelor (0.03-10μM) in citrated PRP. Data represent the mean ± s.e.m. of responses from 4 individuals.

4.3.2 Concentration-ranging of ticagrelor, PAM and aspirin against U46619-induced aggregation

The effect of these anti-platelet agents against U46619-induced platelet aggregation was similar to that seen against ADP. Aspirin had no effect, while both PAM and ticagrelor caused concentration-dependent inhibition of platelet aggregation [figure 4.2]. At 30µM U46619 the log IC_{50} values for both PAM and ticagrelor were similar to those found for the inhibition of aggregations induced by 20µM ADP (log IC_{50} values to U46619: ticagrelor, -6.244; PAM, -5.252). Both $P2Y_{12}$ receptor blockers were equipotent against aggregation responses to all concentrations of U46619.

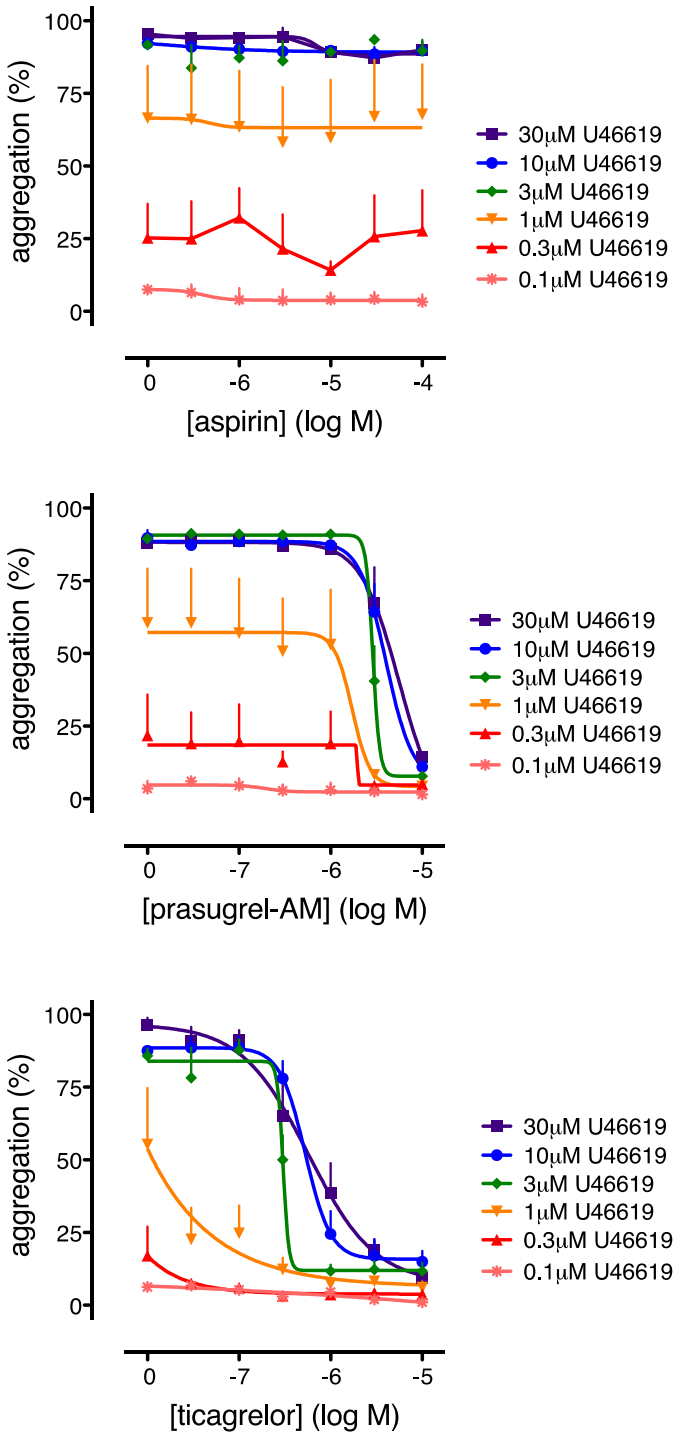


Figure 4.2 The effect of ticagrelor, PAM and aspirin on U46619 induced platelet aggregation.

Concentration dependent inhibition of U46619 (0.1-30μM) induced platelet aggregation by aspirin (0.1-100μM), PAM (0.03-10μM) and ticagrelor (0.03-10μM) in citrated PRP. Data represent the mean ± s.e.m. of responses from 4 individuals.

4.3.3 Concentration-ranging of ticagrelor, PAM and aspirin against arachidonic acid-induced platelet aggregation and TXA_2 production

Aspirin caused a complete, concentration-dependent inhibition of both arachidonic acid-induced platelet aggregation and TXA_2 production (measured as TXB_2 production). Similar inhibition was achieved with both PAM and ticagrelor [figure 4.3] and similar to their differential potencies against ADP and U46619 induced aggregation, ticagrelor was more potent than PAM at inhibiting arachidonic acid-induced aggregation (log IC_{50} values against 0.6mM: ticagrelor, -6.799; PAM, -5.902). Despite the higher potency of ticagrelor, the inhibition of TXA_2 production was similar for both PAM and ticagrelor, and, unlike aspirin, at higher concentrations of arachidonic acid the inhibition was incomplete [figure 4.3].

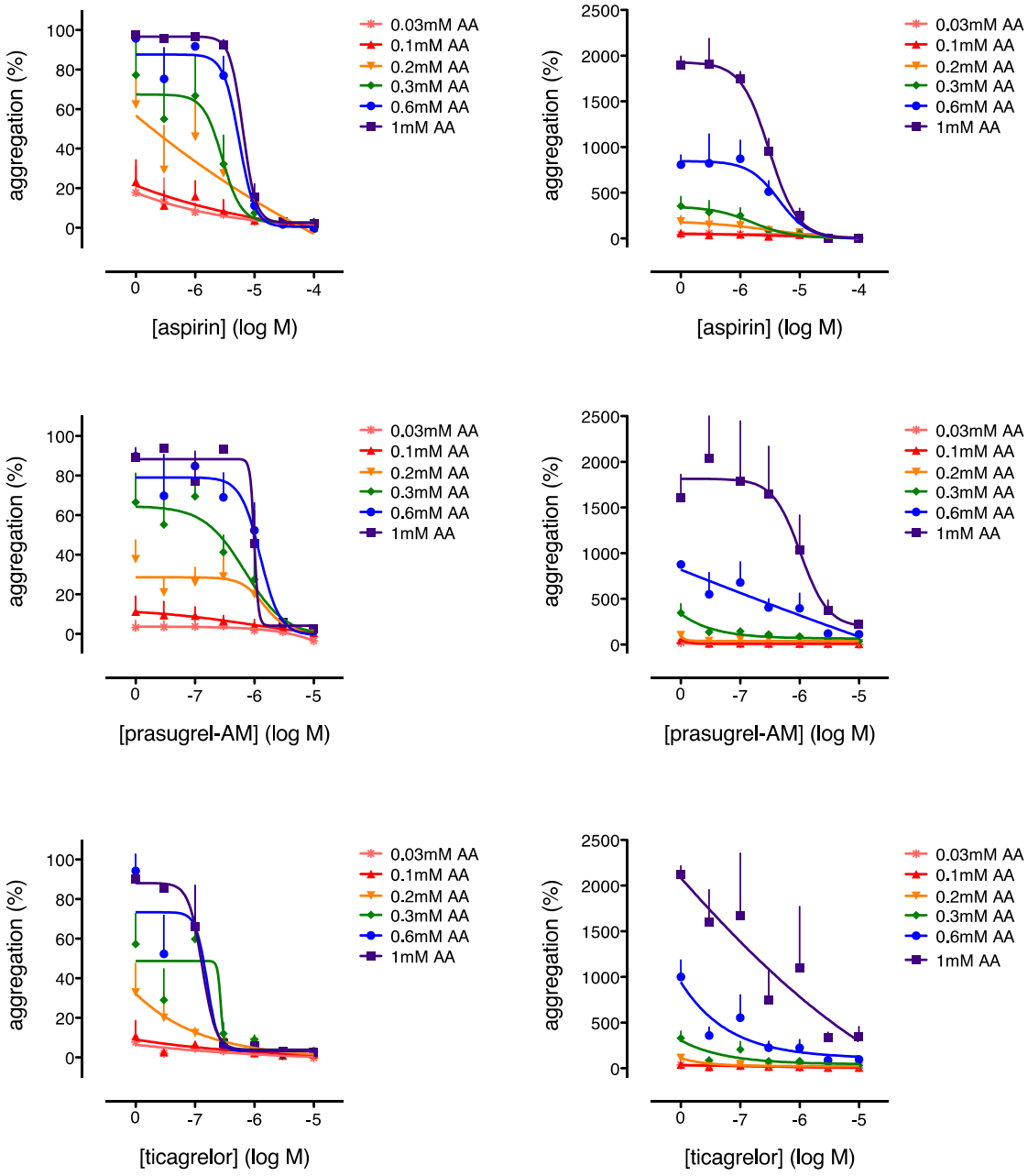


Figure 4.3 Arachidonic acid induced platelet aggregation and TXB_2 production in the presence of ticagrelor, PAM and aspirin.

Concentration dependent inhibition of arachidonic acid (0.03-1mM) induced platelet aggregation and TXB_2 production by aspirin (0.1-100µM), PAM (0.03-10µM) and ticagrelor (0.03-10µM) in citrated PRP. Data represent the mean ± s.e.m. of responses from 4 individuals.

4.3.4 The effect of aspirin, PAM and ticagrelor, at varied concentrations, alone and in combination, on platelet aggregation

In order to examine the effects of aspirin on both complete and partial $P2Y_{12}$ receptor blockade concentrations of PAM and ticagrelor were selected based upon their inhibitory effects against a strong ADP stimulus, 20μM ADP. Concentrations approximating the IC_5, IC_{10}, IC_{50} and IC_{90} were selected to give a broad range of $P2Y_{12}$ receptor blockade. For ticagrelor these concentrations were 0.03μM, 0.1μM, 0.3μM and 3μM; for PAM, 0.5μM, 1μM, 2μM and 10μM. Initially, these concentrations were used either alone or in combination with 30μM aspirin, which reflects the peak plasma concentration following ingestion of a low, 75-100mg, aspirin dose (see chapter 3). However, depending on geographical location, it is not uncommon for higher, 300-325mg, aspirin doses to be prescribed and so the effect of 120μM aspirin was studied.

4.3.4.1 The effect of 30μM aspirin on platelet aggregation alone and in the presence of weak $P2Y_{12}$ receptor blockade

In the presence of weak $P2Y_{12}$ receptor blockade by ticagrelor (0.03μM or 0.1μM) platelet aggregation responses to arachidonic acid, ADP, collagen, adrenaline and U46619 were only weakly inhibited, or in the case of TRAP-6 amide, unaffected [figure 4.4]. Whilst aspirin had a very strong effect against arachidonic acid-induced aggregation it had only a moderate effect against collagen and adrenaline-induced responses, and little to no effect against other agonists. In all cases, the addition of aspirin to weak $P2Y_{12}$ receptor blockade had an additional anti-aggregatory effect that, with the exception of arachidonic acid, was greater than the effects of aspirin alone. The additional effect of aspirin in the presence of weak $P2Y_{12}$ receptor blockade was particularly evident against adrenaline-induced aggregation [figure 4.4].

The effects of weak $P2Y_{12}$ receptor blockade by PAM (0.5µM or 1µM) were very similar to the effects of ticagrelor, whereas aspirin alone had a moderate effect on aggregation induced by collagen and a strong effect on aggregations induced by arachidonic acid or adrenaline. Similarly to ticagrelor, the addition of aspirin furthered the anti-aggregatory effect of low concentrations of PAM against aggregations induced by arachidonic acid, collagen or adrenaline [figure 4.5].

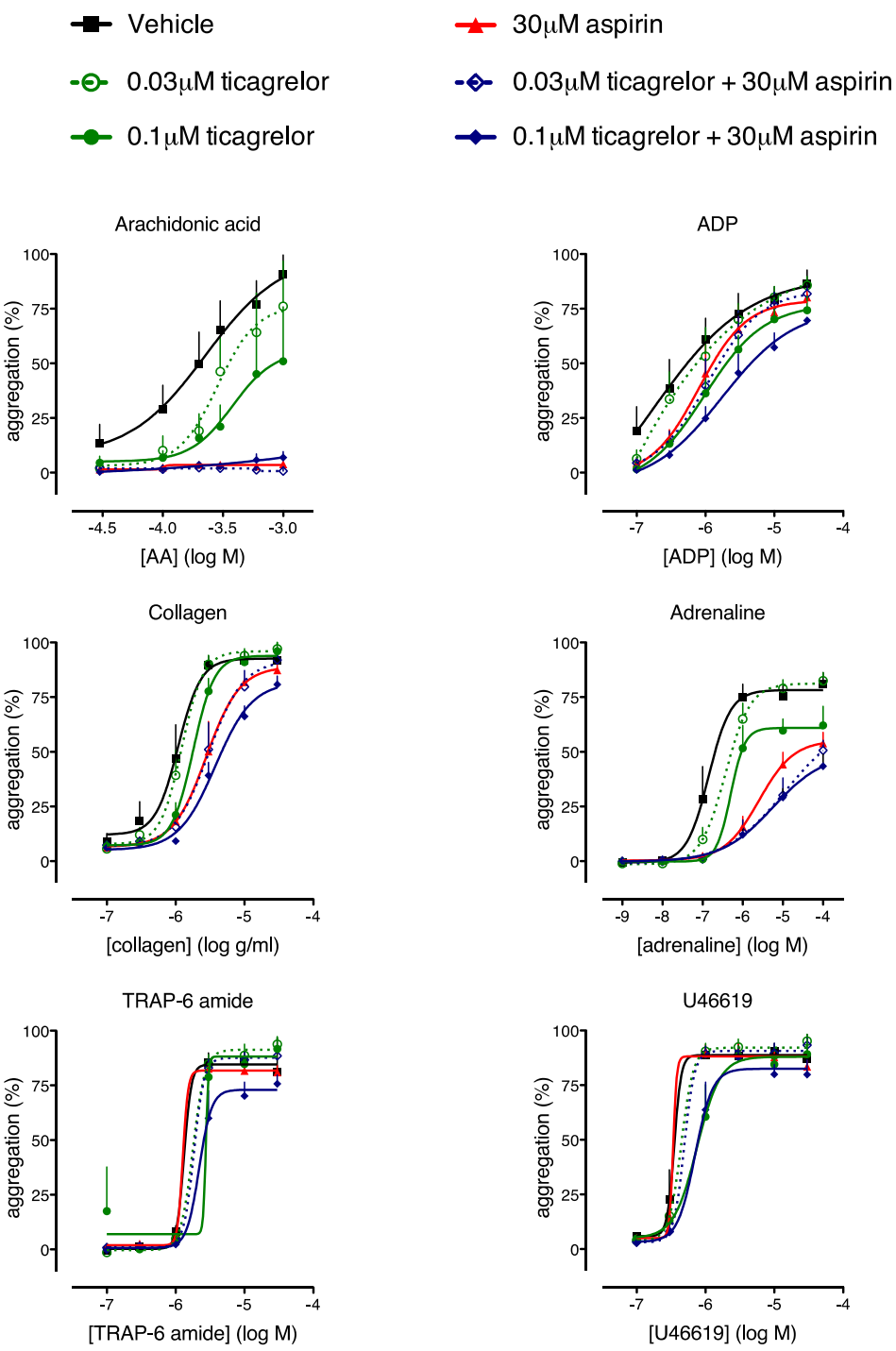


Figure 4.4 Platelet aggregation to six agonists in the presence of aspirin and partial $P2Y_{12}$ inhibition by ticagrelor.

Platelet aggregation in citrated PRP induced by arachidonic acid (0.03-1mM), ADP (0.1-30μM), collagen (0.1-30μg/ml), adrenaline (0.001-100μM), TRAP-6 amide (0.1-30μM) and U46619 (0.1-30μM) in the presence of aspirin 30μM and/or ticagrelor (0.03 or 0.1μM). Data represent the mean ± s.e.m. of responses from 4 individuals.

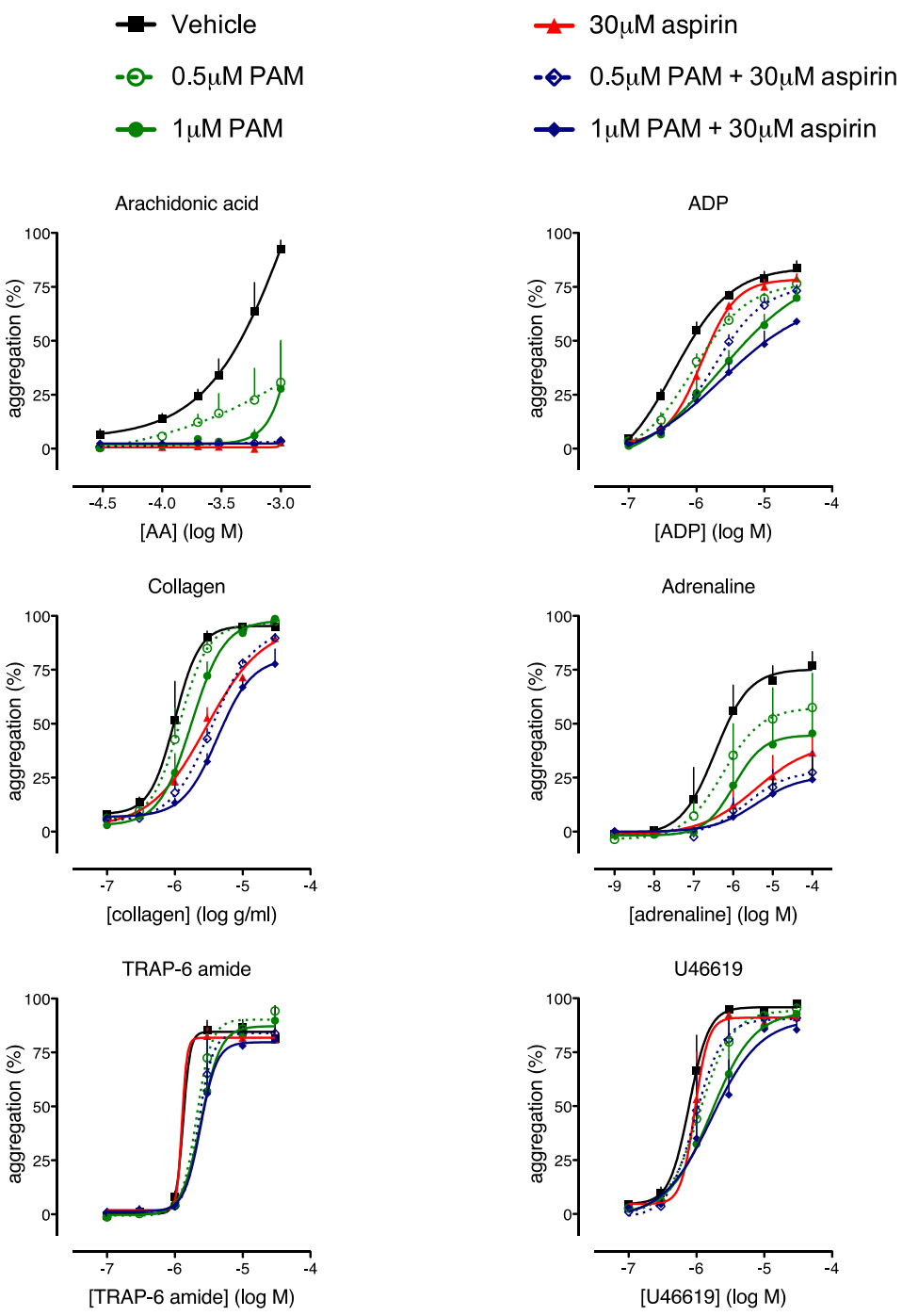


Figure 4.5 Platelet aggregation to six agonists in the presence of aspirin and partial $P2Y_{12}$ inhibition by PAM.

Platelet aggregation in citrated PRP induced by arachidonic acid (0.03-1mM), ADP (0.1-30μM), collagen (0.1-30μg/ml), adrenaline (0.001-100μM), TRAP-6 amide (0.1-30μM) and U46619 (0.1-30μM) in the presence of aspirin 30μM and/or PAM (0.5 or 1μM). Data represent the mean ± s.e.m. of responses from 4 individuals.

4.3.4.2 The effect of 30µM aspirin on platelet aggregation and TXA_2 production alone and in the presence of strong $P2Y_{12}$ receptor blockade

At higher concentrations, (0.3µM and 3µM) ticagrelor had a broad anti-platelet effect. Arachidonic acid-induced aggregation was completely inhibited by even a moderate concentration of ticagrelor (0.3µM) and by aspirin alone. ADP, TRAP-6 amide and U46619-induced aggregations were moderately inhibited by 0.3µM ticagrelor, with no further effect with the addition of aspirin. Collagen and adrenaline-induced responses were also moderately inhibited, although for these agonists aspirin enhanced the effects of 0.3µM ticagrelor. The strong effect of 3µM ticagrelor against arachidonic acid, ADP, collagen, adrenaline, TRAP-6 amide and U46619 was not enhanced by the addition of 30µM aspirin, with the exception of collagen, where aspirin had a very small additional inhibitory effect that was not statistically significant [figure 4.6]. These results were consistent with a partial reduction in collagen-induced TXA_2 production by 0.3µM ticagrelor that was completely inhibited by either the addition of aspirin or increasing the concentration of ticagrelor [figure 4.7].

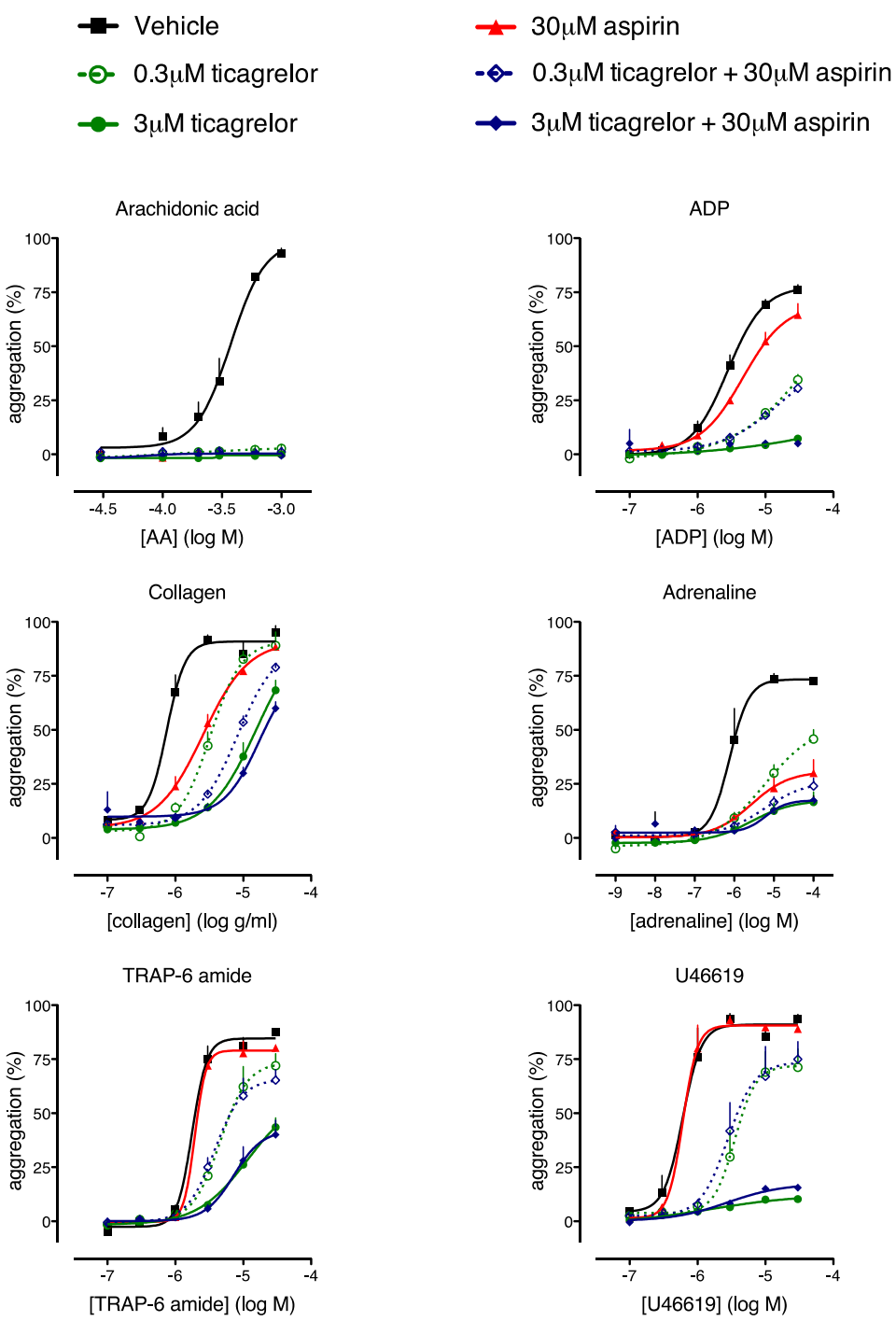


Figure 4.6 Platelet aggregation to six agonists in the presence of aspirin and strong $P2Y_{12}$ inhibition by ticagrelor.

Platelet aggregation in citrated PRP induced by arachidonic acid (0.03-1mM), ADP (0.1-30μM), collagen (0.1-30μg/ml), adrenaline (0.001-100μM), TRAP-6 amide (0.1-30μM) and U46619 (0.1-30μM) in the presence of aspirin 30μM and/or ticagrelor (0.3 or 3μM). Data represent the mean ± s.e.m. of responses from 4 individuals.

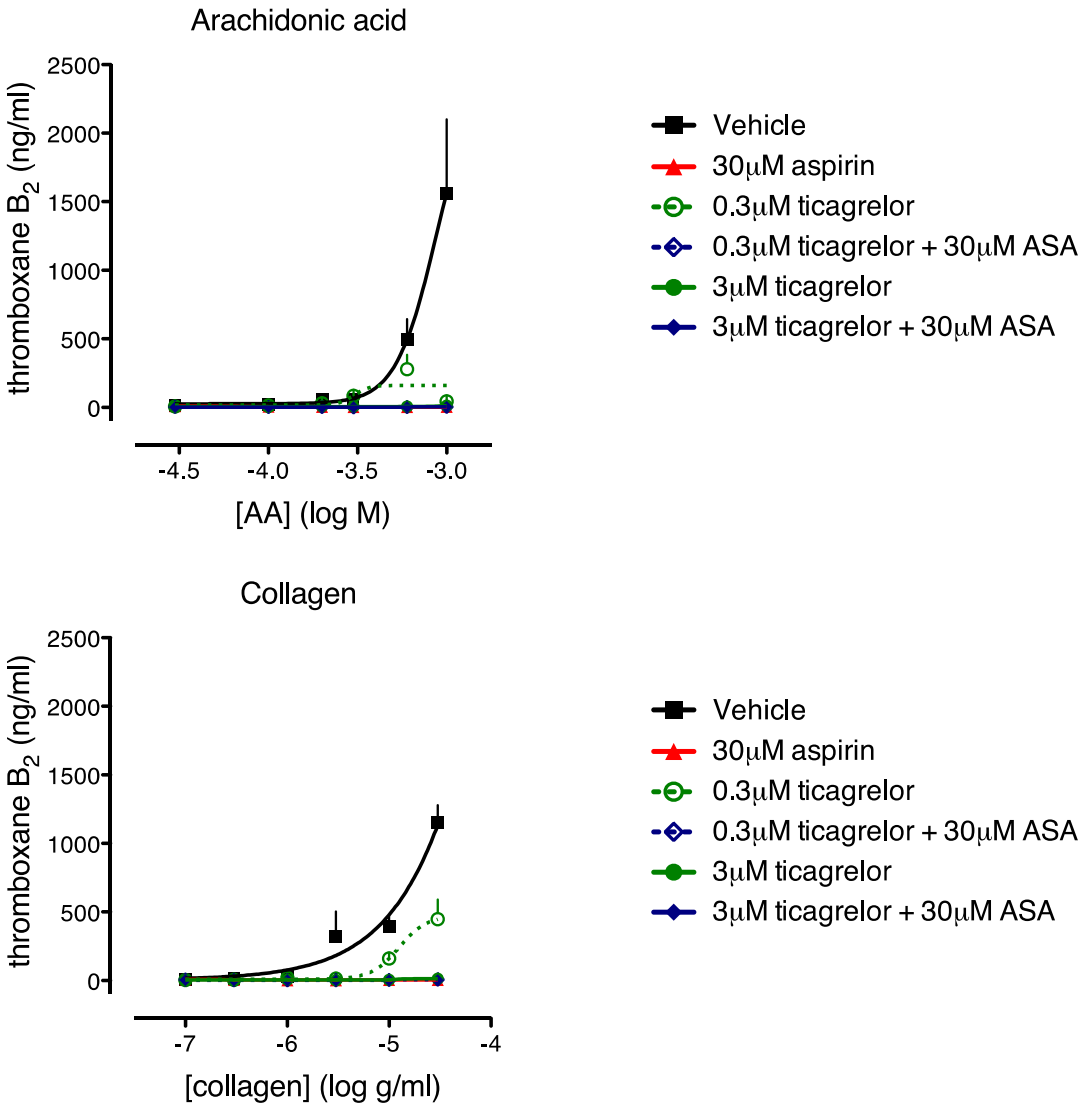


Figure 4.7 TXB_2 production in the presence of aspirin and strong $P2Y_{12}$ inhibition by ticagrelor.

TXB_2 production in citrated PRP after platelet aggregation induced by arachidonic acid (0.03-1mM) and collagen (0.1-30μg/ml) in the presence of aspirin 30μM and/or ticagrelor (0.3 or 3μM). Data represent the mean ± s.e.m. of responses from 4 individuals.

At equivalent inhibitory concentrations (against 20µM ADP-induced aggregation) PAM had very similar effects to ticagrelor. At 2µM, the effects of PAM were only enhanced by the addition of aspirin in the case of collagen and adrenaline-induced aggregation, although at 10µM PAM the only additional effect of aspirin was to cause a very small non-significant reduction in collagen-induced aggregation [figure 4.8]. This was associated with a further reduction in collagen-induced TXA_2 production when aspirin was added to PAM [figure 4.9].

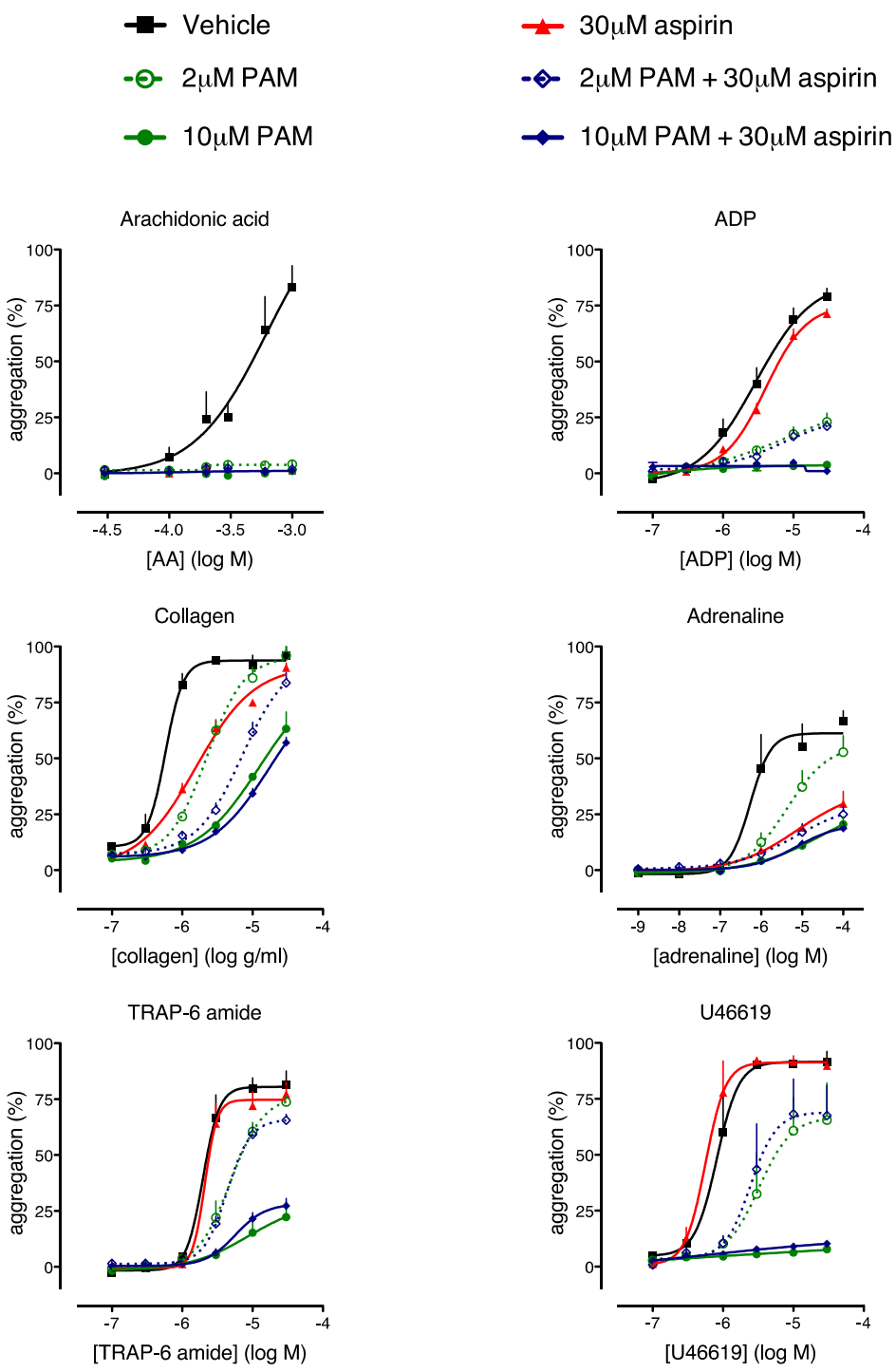


Figure 4.8 Platelet aggregation to six agonists in the presence of aspirin and strong $P2Y_{12}$ inhibition by PAM.

Platelet aggregation in citrated PRP induced by arachidonic acid (0.03-1mM), ADP (0.1-30μM), collagen (0.1-30μg/ml), adrenaline (0.001-100μM), TRAP-6 amide (0.1-30μM) and U46619 (0.1-30μM) in the presence of aspirin 30μM and/or PAM (2 or 10μM). Data represent the mean ± s.e.m. of responses from 4 individuals.

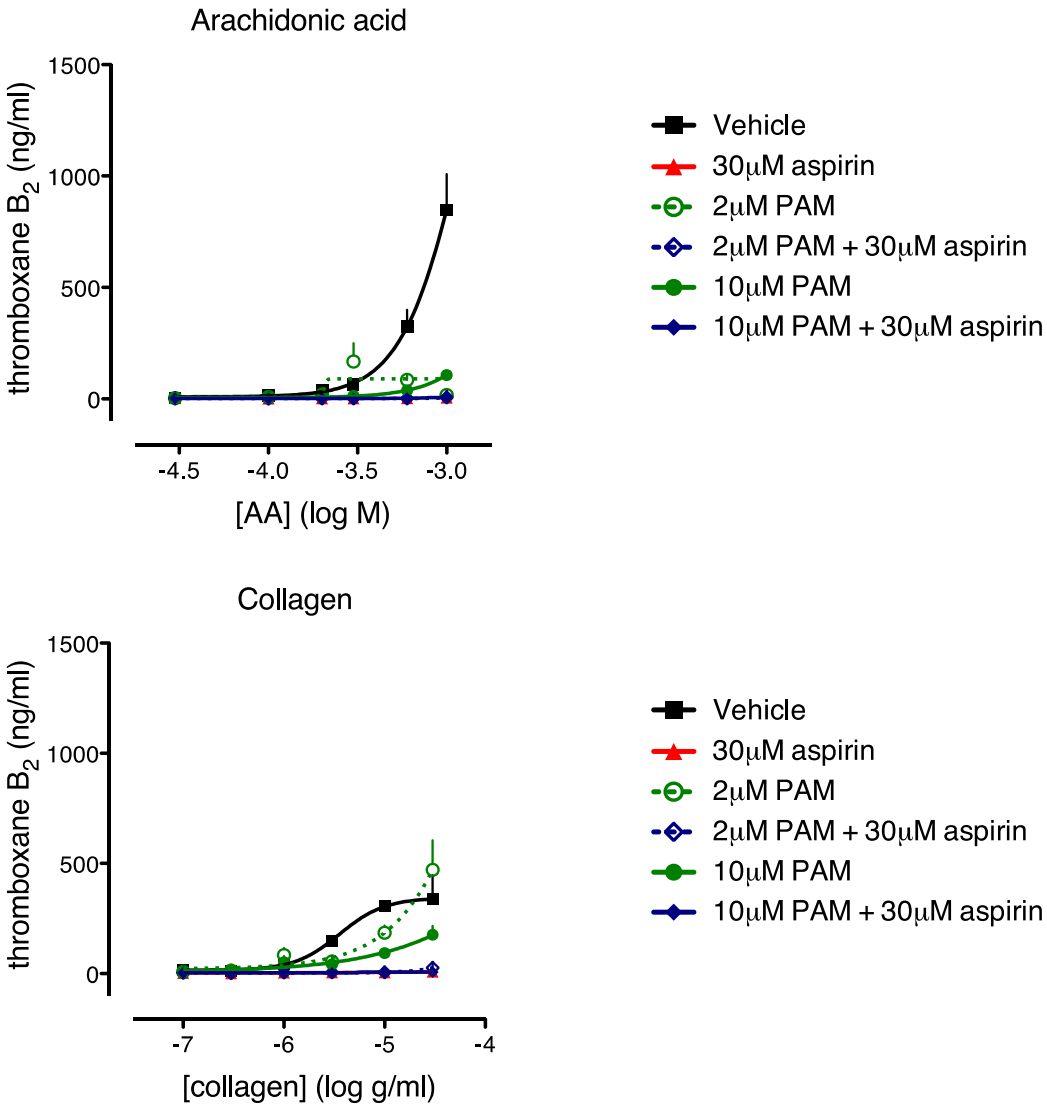


Figure 4.9 TXB_2 production in the presence of aspirin and strong $P2Y_{12}$ inhibition by PAM.

TXB_2 production in citrated PRP after platelet aggregation induced by arachidonic acid (0.03-1mM) and collagen (0.1-30μg/ml) in the presence of aspirin 30μM and/or PAM (2 or 10μM). Data represent the mean ± s.e.m. of responses from 4 individuals.

4.3.4.3 The effect of 120μM aspirin on platelet aggregation alone and in the presence of strong $P2Y_{12}$ receptor blockade

Increasing the aspirin concentration to 120μM did not enhance the anti-aggregatory effect of either aspirin alone or aspirin in the presence of moderate or high concentrations of ticagrelor [figures 4.6 and 4.10]. The same was true for this high concentration of aspirin in the presence of $P2Y_{12}$ receptor blockade by PAM. 120μM aspirin enhanced the inhibition of collagen and adrenaline-induced aggregation by 2μM PAM, although the only additional effect to 10μM PAM was a very small enhancement of collagen-induced aggregation, which did not exceed the effects of 30μM aspirin added to 10μM PAM [figures 4.8 and 4.11].

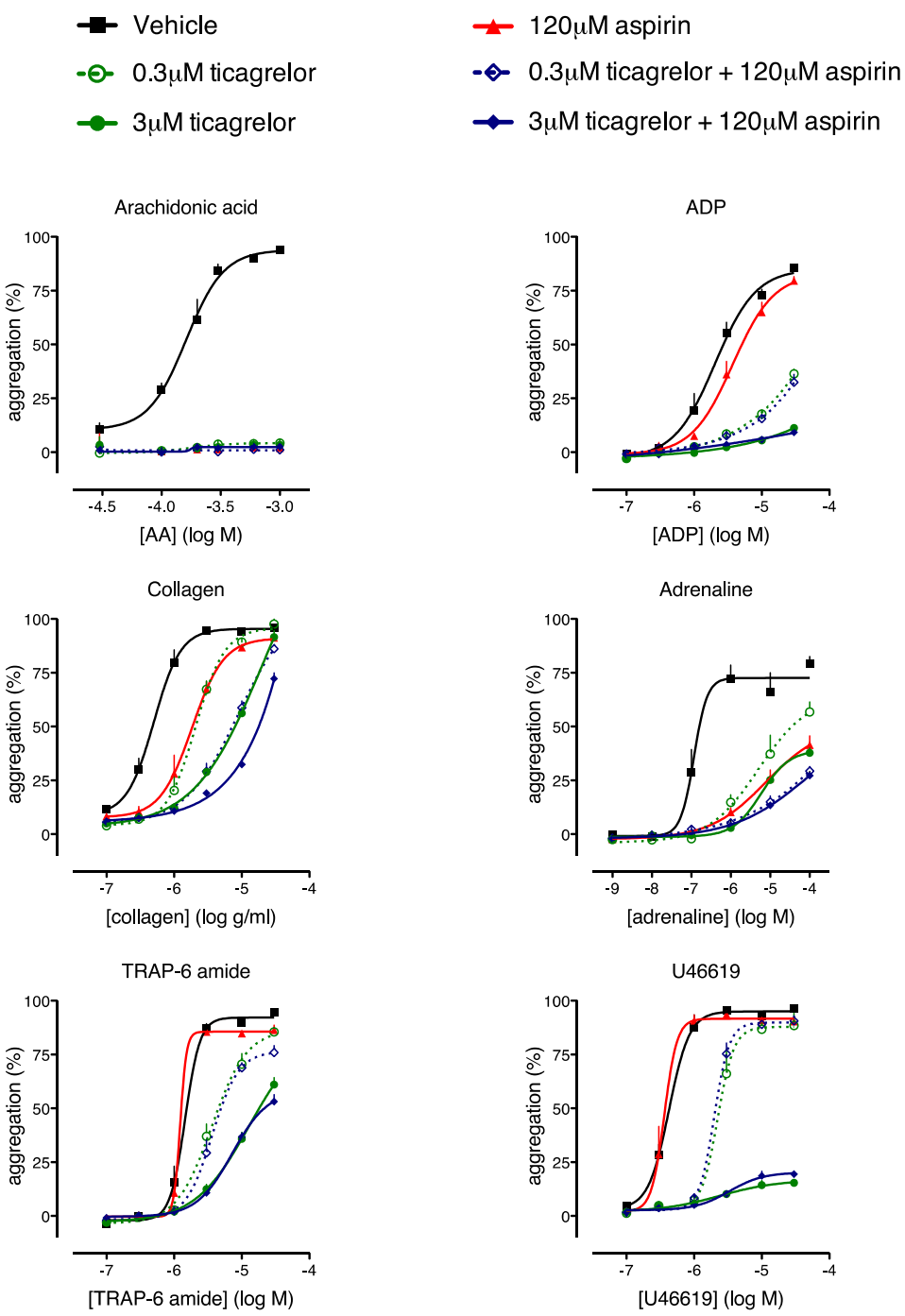


Figure 4.10 Platelet aggregation to six agonists in the presence of 120μM aspirin and strong $P2Y_{12}$ inhibition by ticagrelor.

Platelet aggregation in citrated PRP induced by arachidonic acid (0.03-1mM), ADP (0.1-30μM), collagen (0.1-30μg/ml), adrenaline (0.001-100μM), TRAP-6 amide (0.1-30μM) and U46619 (0.1-30μM) in the presence of aspirin 120μM and/or ticagrelor (0.3 or 3μM). Data represent the mean ± s.e.m. of responses from 4 individuals.

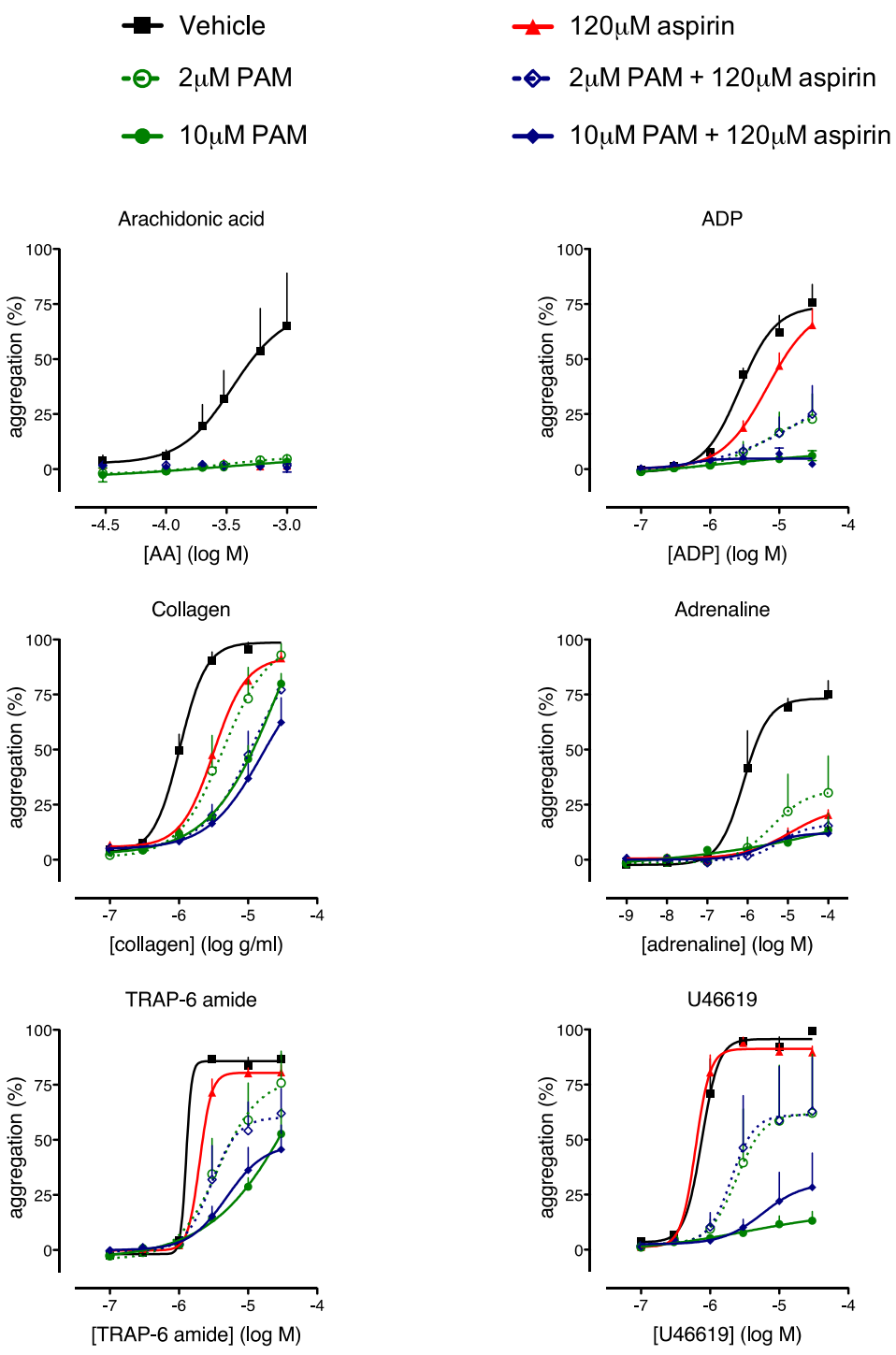


Figure 4.11 Platelet aggregation to six agonists in the presence of 120μM aspirin and strong $P2Y_{12}$ inhibition by PAM.

Platelet aggregation in citrated PRP induced by arachidonic acid (0.03-1mM), ADP (0.1-30μM), collagen (0.1-30μg/ml), adrenaline (0.001-100μM), TRAP-6 amide (0.1-30μM) and U46619 (0.1-30μM) in the presence of aspirin 30μM and/or PAM (2 or 10μM). Data represent the mean ± s.e.m. of responses from 4 individuals.

4.4 Summary

In this chapter I have investigated the anti-platelet effects of cyclo-oxygenase inhibition by aspirin in the presence of partial and complete $P2Y_{12}$ receptor blockade, using two different $P2Y_{12}$ inhibitors, PAM and ticagrelor. Initial experiments showed that ticagrelor was more potent than PAM, and so equivalent concentrations were selected based on inhibitory concentration (IC) values for the inhibition of 20μM ADP-induced aggregation, a $P2Y_{12}$-specific response. At equivalent inhibitory concentrations PAM and ticagrelor had similar anti-platelet effects to a range of agonists, demonstrating that *in vitro* they have a similar mechanism of action. This common mechanism of action of prasugrel, ticagrelor and cangrelor (another investigative agent) has also recently been reported by Iyú et al. [147].

Similarly to the observations of chapter 3, with strong blockade of $P2Y_{12}$ specific responses aspirin contributed little additional anti-platelet effect, despite further reducing agonist-induced TXA_2 production. Even when the aspirin concentration was increased to 120μM there was no further reduction of platelet aggregation compared to that seen with strong $P2Y_{12}$ receptor blockade alone. The exception to this was for collagen where the inhibitory effects of PAM and ticagrelor at concentrations roughly representing IC_{50} values of $P2Y_{12}$-specific responses were enhanced by aspirin. However, this small effect was only seen against aggregation induced by high concentrations of collagen, and was not seen with stronger $P2Y_{12}$ receptor blockade (10μM PAM and 3μM ticagrelor).

With weaker $P2Y_{12}$ receptor blockade the effect of cyclo-oxygenase inhibition was more pronounced. Aspirin increased the anti-platelet effect of $P2Y_{12}$ receptor blockade alone against a range of platelet agonists, supporting a role for the use of aspirin in dual anti-platelet therapy with partial $P2Y_{12}$ receptor blockade.

In vivo, the abundance of cyclo-oxygenases throughout the body means that aspirin can have effects at many extra-platelet sites, whilst the distribution of $P2Y_{12}$ receptors limits the effects of inhibitory ligands primarily to the platelet. When platelet function is maximally inhibited by $P2Y_{12}$ receptor blockade the addition of aspirin will have a very limited platelet effect, although the extra-platelet effects will still exist. For instance, inhibiting endothelial COX function and prostanoid generation, and potentially causing harmful gastrointestinal side effects, which are discussed in more detail in Chapter 8. It is, therefore, important to investigate the contribution of aspirin to *in vivo* anti-platelet therapy with strong $P2Y_{12}$ receptor blockade. A small clinical study to address this is presented in Chapter 5.

As highlighted in Chapter 3, the effects of $P2Y_{12}$ receptor blockade in the 96-well plate model of platelet aggregation differ to those seen in traditional LTA. These differences have been further investigated in Chapter 6 of this thesis.

In vitro the choice of compound to generate strong $P2Y_{12}$ receptor blockade appears to be unimportant although *in vivo* each drug may have quite a different pharmacodynamic profile, which may affect the results of clinical outcome studies, and this is explored in more detail in Chapter 8.

Chapter 5: Aspirin as an anti-platelet agent in healthy volunteers taking prasugrel

5.1 Introduction

The evolution of dual-antiplatelet therapy has resulted in the use of clopidogrel and aspirin as the standard therapy for those at high risk of thrombotic vascular events. With emerging alternatives to clopidogrel, such as prasugrel and ticagrelor, comparator outcome studies have been designed to substitute clopidogrel for a novel drug whilst both groups also receive aspirin. However, the differential effects of aspirin in the presence of either weak or strong $P2Y_{12}$ receptor blockade (presented in Chapter 4 of this thesis) suggest that the rationale of aspirin use with either prasugrel or ticagrelor may be diminished. Most clinical outcome studies have assessed the additional effects of a $P2Y_{12}$ receptor blocker to aspirin alone and this study design does not measure the contribution of aspirin to the clinical effect. In this chapter I present a small clinical study that aimed to investigate whether or not aspirin increased the anti-platelet effect achieved with administration of a strong $P2Y_{12}$ receptor blocker alone. To answer this question, the $P2Y_{12}$ receptor blocker was first administered alone, and then in combination with aspirin. As ticagrelor was not available for routine clinical use at the time of this study, prasugrel was used.

In addition to the effects on platelet aggregation this study aimed to investigate the effects of $P2Y_{12}$ receptor blockade alone and in combination with aspirin on other markers of vascular function, such as urinary prostanoid metabolites. Urinary levels of 2,3-dinor-6-keto $PGF_{1\alpha}$ (PGI-M) and 11-dehydro-TXB_2 (TX-M), are both elevated in vascular disease [154, 155] and during thrombotic events [156]; and both these markers are sensitive to aspirin [53]. The whole body effects of both drugs were also investigated by the use of metabolonomic analysis.

5.2 Methods

5.2.1 Ethics and consent

The study was approved by the St Thomas's Hospital Research Ethics Committee (Ref. 07/Q0702/24), conducted according to the Declaration of Helsinki and all volunteers gave written informed consent prior to entering the study.

5.2.2 Study participants

Nine healthy male volunteers aged 18–40 years were recruited and participated in the study. The volunteers' health statuses were determined through their medical histories and physical examination including blood pressure, pulse rate, blood chemistry and urinalysis. Volunteers with normal clinical profiles were included in the study.

5.2.2 Study protocol

Prior to starting this 21 day study, all volunteers had abstained from aspirin, non-steroid anti-inflammatory drugs, paracetamol or any other anti-platelet therapy for 14 days. The volunteers received a 60mg loading dose of prasugrel on day 1 of the study and a maintenance dose of 10mg prasugrel per day on days 2-21. On day 8 of the study, volunteers began taking 75mg aspirin and on day 15 the aspirin dose was increased to 300mg per day until day 21. Compliance was assessed by interview at each study visit. Blood and urine samples were collected on day 0, prior to commencing drug treatment, and on days 7, 14 and 21 of treatment. On overview of the study protocol is shown in figure 5.1.

5.2.3 Blood collection and preparation of PRP/PPP

Blood was collected by venepuncture into tri-sodium citrate, as described in section

2.3 and PRP/PPP was prepared as described in section 2.4. All experiments were completed within 2h of blood collection.

5.2.4 VerifyNow™ $P2Y_{12}$ and aspirin cartridge assays

Blood was collected by venepuncture into 2ml partial fill vacuum tubes containing tri-sodium citrate (3.2% final concentration). VerifyNow™ assays for effects of $P2Y_{12}$ receptor blockade and aspirin activity were performed in accordance with the cartridge package inserts.

5.2.5 96-well plate light transmission aggregometry

Aggregation was determined, as described in section 2.5, with ADP (0.1-30µM), arachidonic acid (0.03-1mM), Horm collagen (0.1-30µg/mL), adrenaline (0.001-100µM), TRAP-6 amide (0.1-30µM), and the stable TXA_2-mimetic U46619 (0.1-30µM). Graphs shown are for aggregation responses at 16min.

5.2.6 PFA-100 cartridge assays

The platelet function analyzer 100 (PFA-100; Siemens, Deer- field, IL, USA) was used to determine the closure time of both collagen/ADP and collagen/adrenaline cartridges. An 850µL sample of citrated whole blood was dispensed into each cartridge before initiation of testing.

5.2.7 Thromboxane B_2 assay

Plasma TXB_2 levels were determined after platelet aggregation using the overnight incubation assay described in section 2.8.2.

5.2.8 Urine collection and storage

A fresh mid-stream urine sample was collected into sterile containers and the preservative chlorhexidine-propyl gallate (1:1, 20% (wt/v) aqueous chlorhexidine digluconate and 20% (wt/v) n-propyl gallate in methanol) added at 1:500 parts. Samples were then stored at -40ºC for later assay of urinary prostanoid metabolites.

5.2.9 Urinary prostanoid metabolite assays

PGI-M (2,3-dinor-6-keto $PGF_{1\alpha}$) and TXB-M (11-dehydro-TXB_2) were quantified using stable isotope dilution assays with gas chromatography/mass spectrometry, as previously described [157]. These experiments were performed by Ms. Sarah Thomas and Dr. Ginger Milne at Vanderbilt University School of Medicine.

5.2.10 Metabolomic analysis

A metabolomic analysis of urine samples was performed using gas chromatography/mass spectrometry and liquid chromatography/mass spectrometry platforms by Metabolon Inc., Durham, NC, USA. This analysis included 301 named biochemical compounds. Raw data were normalised to osmolality of each sample and then re-scaled to have a median equal to 1. These experiments and analyses were performed by staff at Metabolon Inc.

5.2.11 Statistical analysis

All statistical analyses were conducted using GraphPad Prism v5 (GraphPad Software Inc, CA, USA). Agonist concentration-response curves were plotted and analysed according to the four parameter logistic equation: Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))

Concentration-response curves were compared by two way ANOVA and Bonferroni

post-tests. Metabolonomic samples were compared by matched t-tests. In each case, $p \leq 0.05$ was taken as a significant change.

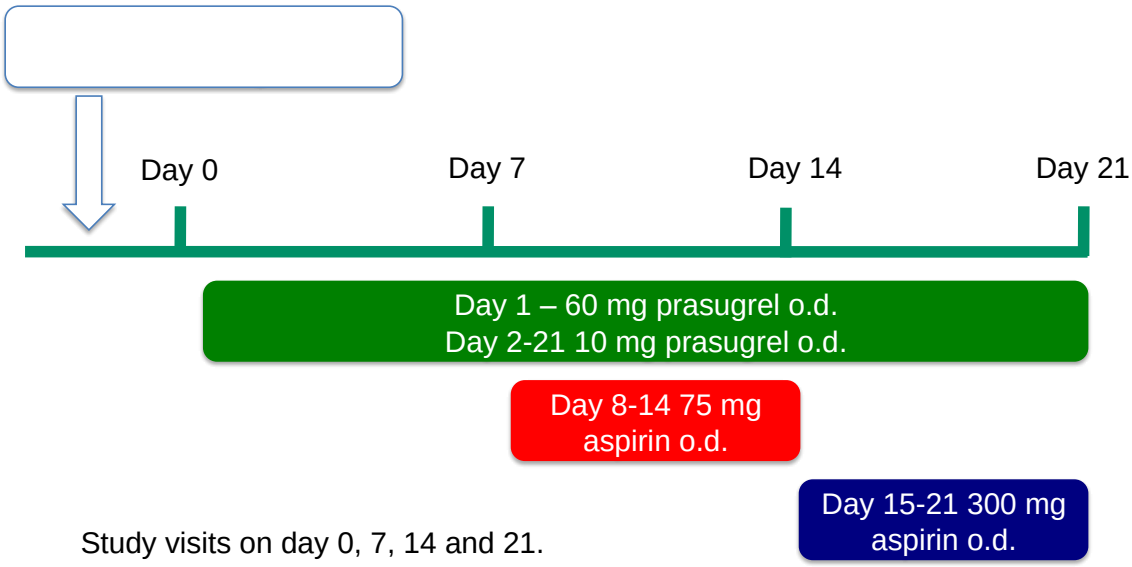


Figure 5.1 Healthy volunteers study timeline.

Study participants were screened within 14 days prior to commencing the study. 9 participants were enrolled in this study. At the first study visit, day 0, samples were taken to provide a pre-drug baseline. Prasugrel 60mg was loaded on day 1, and a 10mg daily maintenance dose was taken on days 2-21. Aspirin was started on day 8, at 75mg daily for 7 days, and the dose increased to 300mg daily on day 15 for the final 7 days of the study. Participants were instructed to take all drugs at the start of the morning each day. All study visits were conducted during the morning.

5.3 Results

5.3.1 Responses to prasugrel alone and in combination with aspirin in the VerifyNow™ $P2Y_{12}$ and aspirin cartridge assays

To confirm the response of the study subjects to both prasugrel and aspirin the VerifyNow™ cartridge assay system was used. None of the subjects had positive tests for either $P2Y_{12}$ inhibitors or aspirin at baseline [figure 5.2]. With prasugrel alone, 8 of the 9 study participants had greater than 95% inhibition of baseline (day 0) activity on the $P2Y_{12}$ assay, whereas the ninth participant had only 50% inhibition, and these results were maintained with the addition of both low and high dose aspirin [figure 5.2]. Although this subject was identified as an outlier from the group, using Grubb's test, all data relating to this subject were included in further analyses. After the addition of low dose aspirin (75mg), all subjects were identified as aspirin-responders, and these responses were maintained at the higher dose of aspirin (300mg) [figure 5.2].

5.3.2 Effects of prasugrel alone and in combination with aspirin on ex vivo platelet aggregation

Platelet aggregation to arachidonic acid, ADP and U46619 was strongly inhibited at all concentrations by prasugrel alone, and prasugrel in combination with aspirin, although there was no statistically significant reduction with the addition of low dose aspirin, and again no change with an increased dose of aspirin [figure 5.3]. Aggregation responses to collagen, adrenaline and TRAP-6 amide were also strongly inhibited by prasugrel alone, and at the highest concentrations of these agonists aspirin did statistically increase the level of inhibition, although this effect was no different with either low or high dose aspirin [figure 5.3].

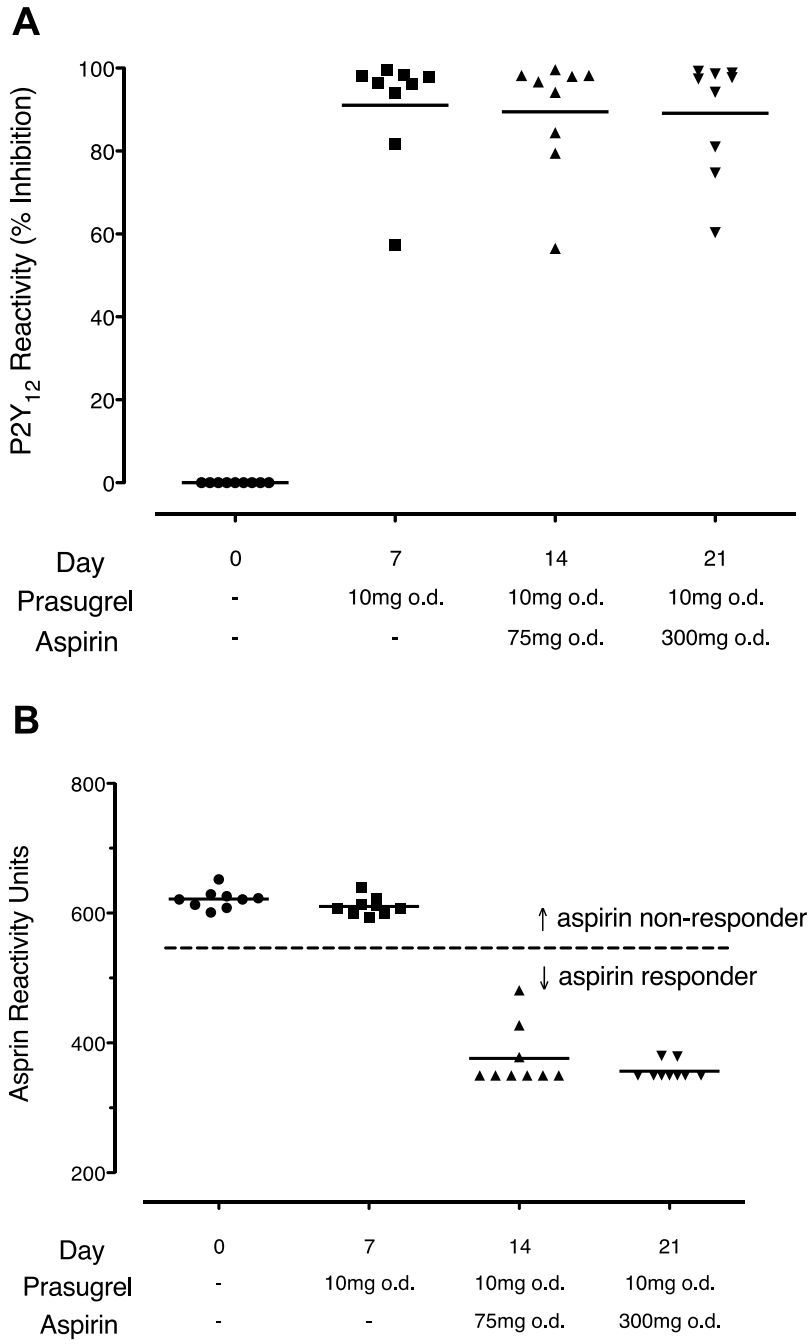


Figure 5.2 Platelet reactivity: VerifyNow™ aspirin and $P2Y_{12}$ cartridge assays.

Panel A; Reported $P2Y_{12}$ reactivity normalised to day 0 values for each of 9 study participants at study days 0, 7, 14 and 21. Panel B; Aspirin Reactivity Units (ARUs) for each of 9 study participants at study days 0, 7, 14 and 21. ARU values less than 550 indicate a response to aspirin (abnormal platelet response to arachidonic acid). In each panel, horizontal lines indicate the group mean and symbols represent values for individual subjects.

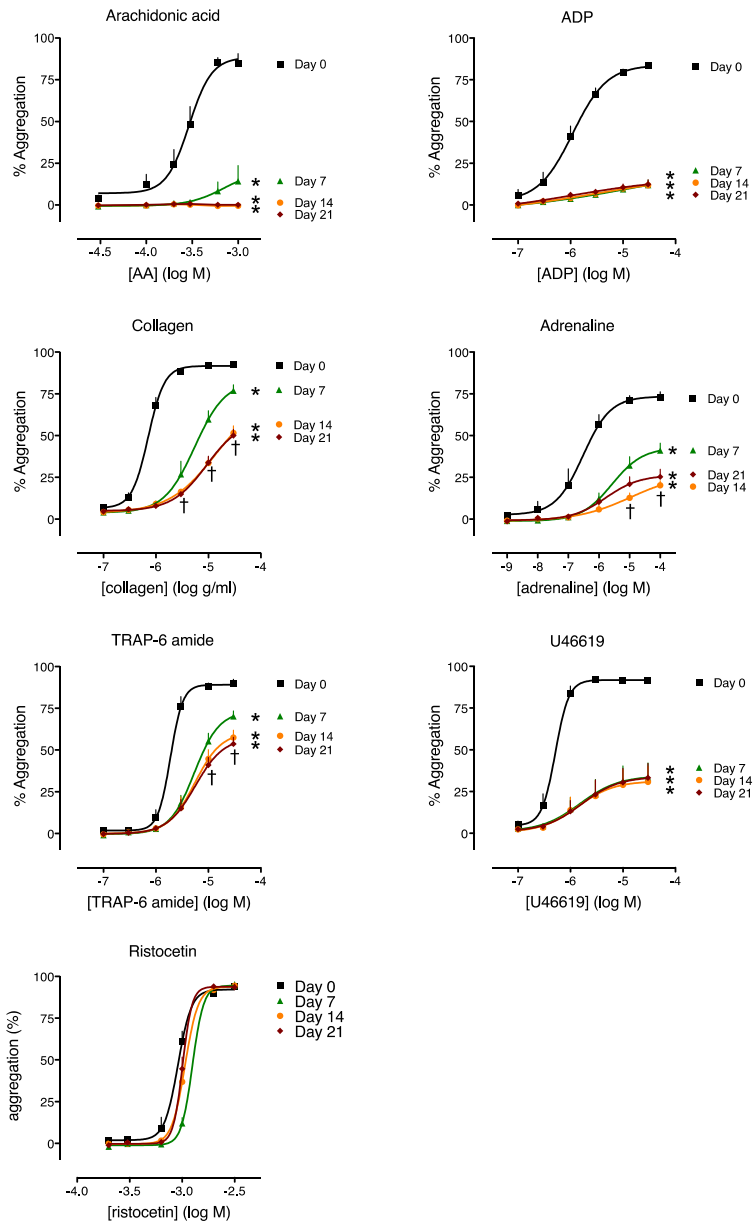


Figure 5.3 Platelet aggregation to six agonists on study days 0, 7, 14 and 21.

Platelet aggregation in citrated PRP induced by arachidonic acid (0.03-1mM), ADP (0.1-30µM), collagen (0.1-30µg/ml), adrenaline (0.001-100µM), TRAP-6 amide (0.1-30µM), U46619 (0.1-30µM) and ristocetin (0.02-3mM) on study days 0 (baseline), 7, 14 and 21. Data represent the mean ± s.e.m. of responses from 9 subjects. * indicates $p<0.05$ by two-way ANOVA compared to baseline; † indicates $p<0.05$ at day 14 and day 21 compared to day 7. No differences occurred between days 14 and day 21 for any agonist ($p>0.05$, two-way ANOVA). Symbols at the end of lines indicate a difference for all concentrations; symbols at individual points indicate a difference at that concentration.

5.3.3 Thromboxane production in response to arachidonic acid and collagen

Prasugrel administration partially reduced the production of TXA_2 in response to both arachidonic acid and collagen by platelets in 96-well aggregometry [figure 5.4]. Co-administration of either dose of aspirin with prasugrel resulted in the complete abolition of any remaining TXA_2 production.

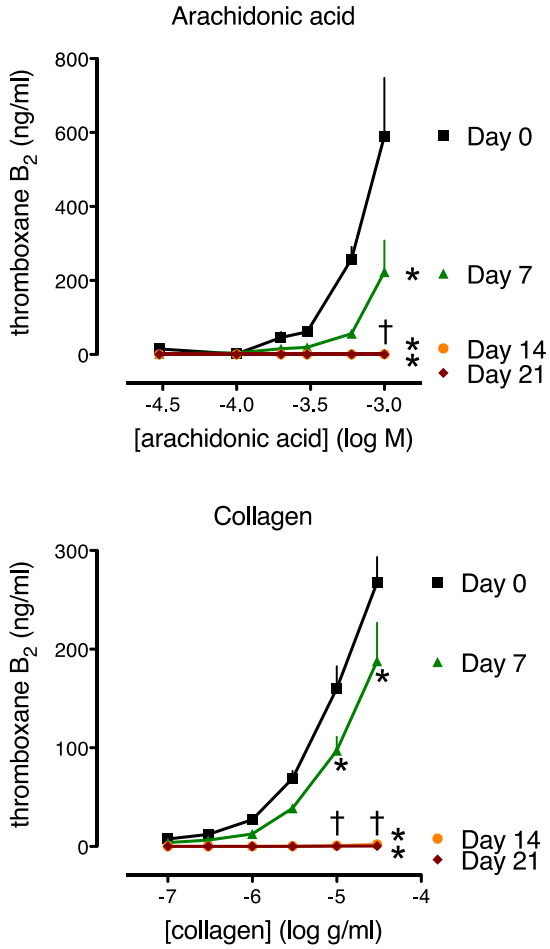


Figure 5.4 TXB_2 production induced by arachidonic acid and collagen on study days 0, 7, 14 and 21.

TXB_2 production in citrated PRP after platelet aggregation induced by arachidonic acid (0.03-1mM) and collagen (0.1-30µg/ml) study days 0 (baseline), 7, 14 and 21. Data represent the mean ± s.e.m. of responses from 9 individuals. * indicates $p<0.05$ compared to baseline, by two-way ANOVA. † indicates $p<0.05$ at day 14 and day 21 compared to day 7. Symbols at the end of lines indicate a difference for all concentrations; symbols at individual points indicate a difference at that concentration.

5.3.4 PFA-100 collagen/ADP and collagen/adrenaline cartridge closure time

Both the collagen/ADP and collagen/adrenaline cartridge closure times were within the normal ranges before the study commenced, 61-104 seconds for collagen/ADP and 74-146 seconds for collagen/adrenaline [figure 5.5]. At day 7, the collagen/ADP closure time was prolonged, and remained so at the later time points [figure 5.5]. The collagen/adrenaline closure time was unaffected by prasugrel alone and remained within the normal range until day 14, after the addition of low dose aspirin, at which point it exceeded the limit of the assay (aperture closure did not occur within 300 seconds). Interestingly, whilst still prolonged, after the higher dose of aspirin (day 21) the closure time for each cartridge tended to be shorter than after low dose aspirin (day 14) [figure 5.5].

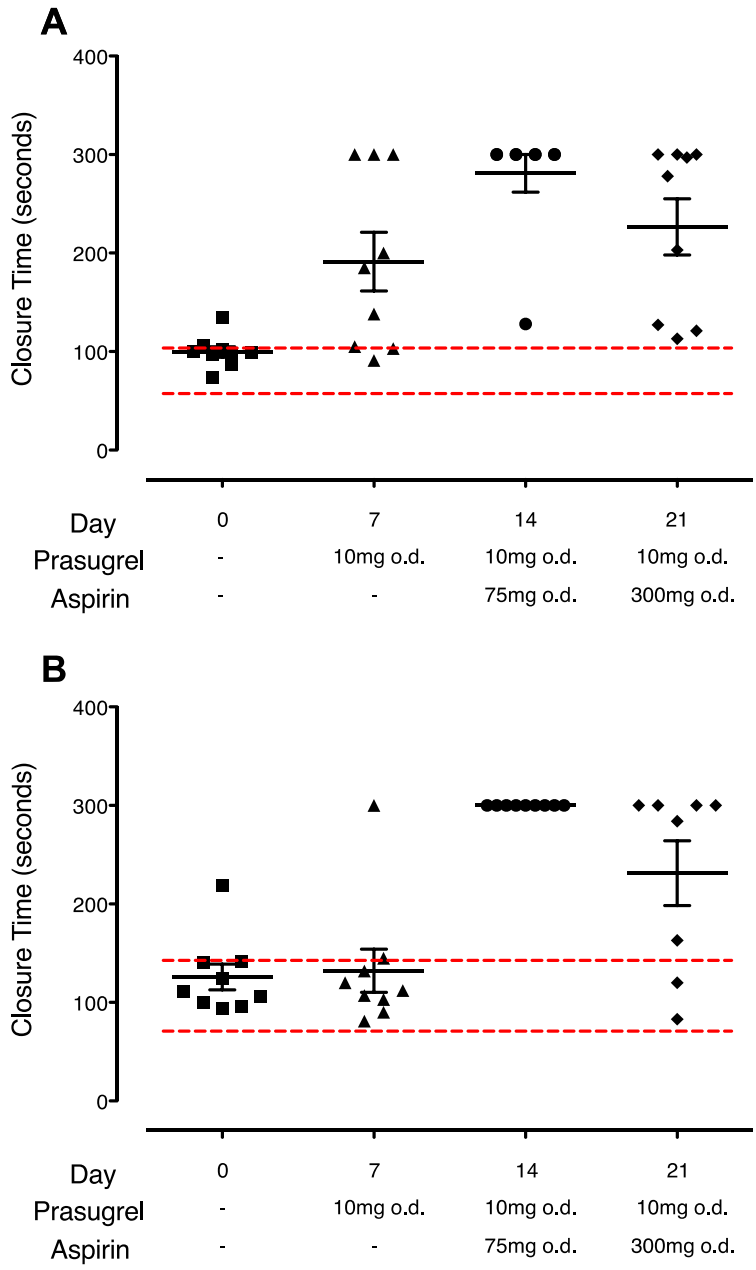


Figure 5.5 Closure time of PFA-100 collagen/ADP and collagen/adrenaline cartridges on study days 0, 7, 14 and 21.

PFA-100 collagen/ADP (Panel A) and collagen/adrenaline (Panel B) cartridge closure time at days 0, 7, 14 and 21. Dashed lines indicate the normal range for closure time of each cartridge: Panel A, collagen/ADP cartridge normal closure time, 61-104 seconds; Panel B, collagen/adrenaline cartridge normal closure time, 74-146 seconds. All data points for collagen/adrenaline closure time at day 14 exceeded 300 seconds, the maximum time period for this assay. Solid horizontal lines and vertical error bars show the mean ± s.e.m. for 9 study participants.

5.3.5 Production of urinary prostanoid metabolites

In addition to reducing platelet derived TXA_2 production, aspirin can, by inhibition of cyclo-oxygenases, reduce the production of vascular PGI_2. Changes in circulatory and vascular prostanoids may be reflected by changes in urinary prostanoid metabolites, which are a measure of 'whole-body' prostanoid production. To assess the changes in urinary prostanoid levels the stable urinary metabolites of thromboxane, 11-dehydro-TXB_2 (TX-M), and PGI_2, 2-3 dinor-6-keto $PGF_{1\alpha}$ (PGI-M), were determined at each time point of the study.

Neither urinary TX-M, nor PGI-M was reduced from baseline values with prasugrel alone. When prasugrel and low dose aspirin were co-administered, the levels of both urinary prostanoid metabolites decreased, although there was no further decrease seen with prasugrel in combination with high dose aspirin [figure 5.6].

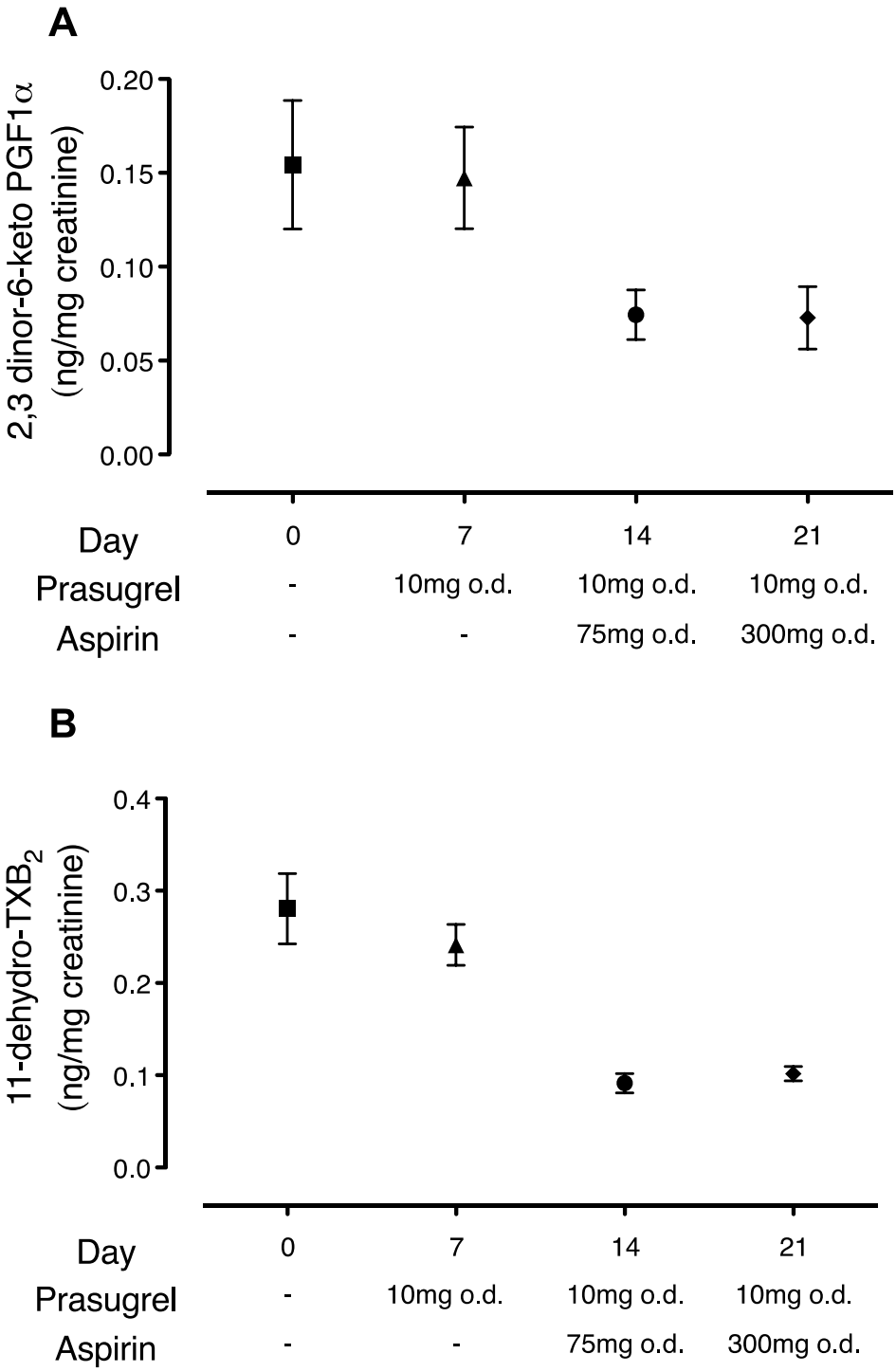


Figure 5.6 Urinary PGI-M and TX-M excretion on study days 0, 7, 14 and 21.

Urinary prostanoid metabolites (PGI-M, upper panel; TX-M, lower panel) corrected against urinary creatinine concentration. Horizontal lines and vertical error bars represent the mean ± s.e.m. for 9 study participants.

5.3.6 Metabolomic analysis of urine samples

A number of biochemical compounds were changed during the time course of the study. Significant, or approaching significant changes were seen in aspirin metabolites, tri-citric acid (TCA) cycle intermediates and branched chain amino acids (BCAAs). The aspirin metabolites salicyluric glucuronide, salicylate and 2-hydroxyhippurate were all significantly increased at day 14 ($p \leq 0.05$), compared to day 0, with a trend towards a further increase at day 21, which only reached significance for salicylate ($p \leq 0.05$, day 14 vs day 21). The compound gentisate, which is both an aspirin and a tyrosine metabolite, was only increased at day 21 [Figure 5.7]. The accumulation of TCA cycle intermediates in urine occurred at day 14, suggesting an inhibition of enzymatic function, which may lead to decreased cellular energy. The BCAAs, which are catabolised to TCA cycle intermediates, isoleucine, leucine and valine were all increased in urine at day 14, compared to either day 7 or day 0.

A non-significant trend for increased urinary ascorbic acid was seen at days 14 (approximately 4-fold higher than baseline) and days 21 (7-fold higher than baseline). These trends were driven by changes in some but not all of the study participants.

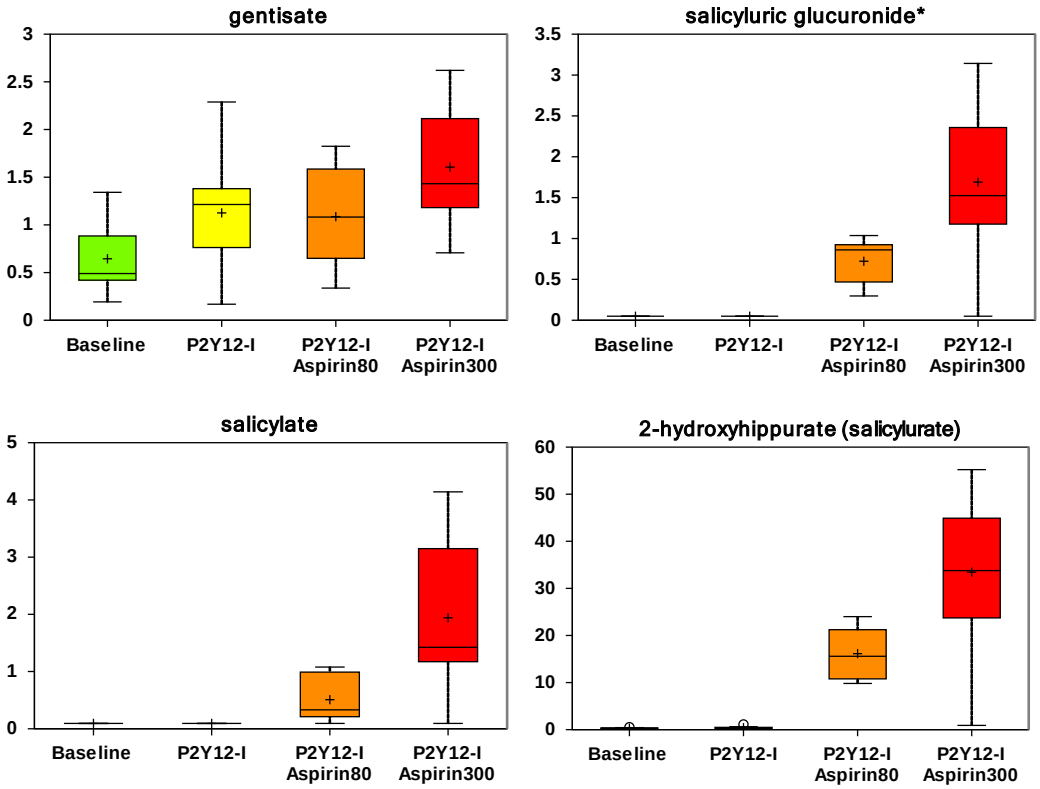


Figure 5.7 Metabolomic analysis: Urinary aspirin metabolites

The four panels above show the urinary levels of select aspirin metabolites at Day 0 (baseline), Day 7 (P2Y12-I), Day 14 (P2Y12-I Aspirin80) and Day 21 (P2Y12-I Aspirin300) for n=9 study participants. Boxes represent the upper quartile (top of box), lower quartile (bottom of box) and median (horizontal line) values. + depicts the mean value and error bars show the minimum and maximum values.

These charts were prepared by Metabolon Inc., Durham, NC, USA.

5.4 Summary

In this study, a standard dosing regime of prasugrel produced a strong anti-platelet effect as assessed by the clinically validated VerifyNow™ cartridge assay system. These data agreed with the results of 96-well plate aggregometry, where ADP-induced aggregation was strongly inhibited. In addition, *ex vivo* platelet aggregation to arachidonic acid, collagen, adrenaline, U46619 and TRAP-6 amide was also inhibited. There was a small additional effect of aspirin on collagen, adrenaline and TRAP-6 induced aggregation, although this only occurred at the highest concentrations and was not furthered by a higher aspirin dose. This was consistent with the measurement of TXB_2 production, which showed that the inhibitory effect of aspirin was maximal at the lower concentration. Prasugrel alone did partially inhibit *ex vivo* platelet TXA_2 production, although as U46619/TP receptor-induced aggregation was largely inhibited it is thought that the contribution of TXA_2 to platelet reactivity was largely diminished.

The results of this clinical study confirm the *in vitro* observations made in Chapter 3, that prasugrel alone has a strong anti-platelet effect that is enhanced by the addition of aspirin only in the case of high concentrations of select agonists. The translation of the *in vitro* model into a clinical study is important as it improves the understanding of how this routinely used dual therapy achieves its effects. It also allows assessment of the effects of these drugs on the platelets natural environment. In this study urinary prostanoid metabolites TX-M and PGI-M were measured as a proxy of whole body prostanoid production [155]. The results show that whilst prasugrel had no effect on either of these metabolites, low dose aspirin reduced both, with no further reduction at a higher aspirin dose. It is unclear how prasugrel can inhibit *ex vivo* platelet thromboxane production without affecting the *in vivo* production, which is believed to be largely platelet derived [19], although this will be discussed in chapter 8. Once

again, it is important to remember that although ‘whole body’ thromboxane production may not have been reduced by prasugrel alone, there is evidence that prasugrel treated platelets are largely insensitive to thromboxane-driven activation.

The PFA-100 cartridge system is intended to model thrombus formation in an injured small artery, anticoagulated blood is drawn through a narrow capillary, generating high rates of shear stress [158]. Blood passes over an exposed, collagen coated, membrane in an aperture of the capillary, to which platelets adhere. The PFA-100 reports the time taken for occlusion of this aperture as the closure time (CT), a prolonged CT being abnormal. This system is sensitive to changes in vWF, and is therefore useful in detecting type 1 von Willebrand disease [159]. However, the use of PFA-100 to detect the effect of anti-platelet drugs is more limited [160]. Variable CTs, both normal and abnormal, have been reported in patients taking aspirin, despite uniform suppression of serum TXA_2, suggesting a strong level of cyclo-oxygenase inhibition [161]. Similarly, variable closure times – normal or prolonged – for both cartridge types are reported with the use of clopidogrel, either alone or with aspirin [162]. This may be explained by the apparent importance of vWF and glycoprotein Ib and IIb/IIIa interactions in determining PFA-100 cartridge responses [150, 162, 163]. In this small study, collagen/ADP CTs tended to be consistently prolonged by prasugrel, although the inhibition of collagen/adrenaline responses was more variable. Given the limitations of this assay it is difficult to draw any conclusions from this data set.

By conducting a metabolomic analysis it has been possible to quantify the effects of aspirin and prasugrel on a wide range of metabolic systems. That said, as the main aim of the study was to quantify the anti-platelet effects of prasugrel alone and in the presence of aspirin the study design did not lend itself to a conclusive metabolomic analysis. Without the inclusion of wash out periods between the different drug

regimens it is not possible to differentiate the effects of time from the effects of each drug treatment. For instance, it is possible that increases in urinary metabolites at day 14 compared to day 7 reflect an adaptive change to prasugrel rather than a change brought about by the addition of low dose aspirin. Clearly in the case of urinary xenobiotics the observed changes are almost certainly due to the administration of aspirin, although, once again it is difficult to distinguish the effects of higher dose from the effects of time. Despite this, these data provide a useful insight of metabolic pathways that may warrant future investigation in specially designed studies.

Increased urinary excretion of ascorbic acid was detected in some but not all individuals after the inclusion of aspirin with prasugrel, and the magnitude of this increase was associated with either the aspirin dose or time on aspirin, although this trend was not significant for the group as a whole. The source of this ascorbic acid (plasma or tissue) is unknown, although in the mid to late 1970s it was reported that aspirin disrupts the binding of ascorbic acid to bovine serum albumin [164] and leads to tissue desaturation of ascorbic acid [165], supporting earlier reports associating aspirin use with decreased platelet [165] and leukocyte [166] levels of this antioxidant. In fact, low leukocyte levels of ascorbic acid had been associated with gastrointestinal bleeding of any cause, and ascorbate was further reduced in cases where the injury was associated with aspirin use, a phenomenon explained by the authors as subclinical scurvy [166]. More recently, the benefits of using ascorbic acid with aspirin have been tested by Bayer Vital GmBH, who reported that fewer gastric lesions develop in healthy subjects taking a preparation of aspirin with ascorbic acid than in those taking aspirin alone [167]. Ascorbic acid with aspirin has also been investigated as a treatment combination after ischaemic stroke, with no loss of clinical benefit, although further outcome studies are needed to identify if this has any long-term functional benefit or if it reduces the incidence/severity of gastrointestinal

bleeding events [168].

In summary, these data suggest that a higher dose of aspirin, in combination with strong $P2Y_{12}$ inhibition did not further the anti-platelet effect. This work furthers the understanding of platelet cyclo-oxygenase activity in the presence of strong $P2Y_{12}$ receptor inhibition and identifies some important avenues for further investigation.

In order to further understand how *in vitro* and *ex vivo* measures characterise the interactions between cyclo-oxygenase and $P2Y_{12}$ pathways of platelet activation it is necessary to fully understand how these measures are derived. This has been assessed in Chapter 6. In addition to further characterisation of platelet function tests it is also important to further the knowledge of how these two pathways contribute to normal function in health and disease. In Chapter 7 I have begun to investigate this by looking at the contribution of COX-1 and COX-2 isoforms to the production of prostanoids in organs relevant to the cardiothoracic system.

Chapter 6: Investigating the mechanisms of traditional light transmission aggregometry

6.1 Introduction

The previous three chapters have presented data in support of the notion that aspirin only enhances the anti-platelet effect of $P2Y_{12}$ receptor blockers in instances of partial, but not complete, $P2Y_{12}$ receptor blockade. The one exception to this generalisation is that aspirin has a very small additional effect on collagen-induced aggregation even in the presence of strong $P2Y_{12}$ receptor blockade. However, this data has been acquired by performing 96-well plate aggregometry and whilst this technique is sensitive for the detection of anti-platelet therapy [143] it is not widely used. Traditional light transmission aggregometry, where light transmission is monitored in a warmed and continuously stirred PRP sample is the predominant technique for assessing platelet reactivity in many research laboratories [150]. As a result, this technique has long been considered a 'gold standard' platelet function test [150, 169]. This distinction may be appropriate for platelet function testing in the diagnosis of bleeding disorders, although in assessing thrombotic risk there has been little agreement between traditional LTA and clinical outcomes [160, 170]. This disagreement between *ex vivo* testing and clinical outcomes suggests that the sensitivity of the test is not sufficient to distinguish effective and ineffective anti-platelet therapy. With regard to platelet aggregation in the presence of strong $P2Y_{12}$ receptor blockade, studies using traditional LTA have reported that collagen-induced aggregation is, at most, moderately inhibited, and that aspirin has a marked additional effect [146].

In order to better understand how anti-platelet therapies alter measurements in traditional LTA, and in turn understand why the results obtained by this method support an additional role of aspirin, I have investigated a number of variables. Typically, traditional LTA is performed at high stir-speeds (1000-1200rpm) for a short, arbitrary period of time, in the range of 1.5 to 5 minutes. In contrast, 96-well plate

aggregometry runs over 16-minutes, with lower speed, intermittent shaking. Therefore, I have investigated the effect of both stir-speed and time on the aggregation responses in traditional LTA; performing aggregation studies at stir-speeds between 100 and 1200rpm for periods of 5 to 20 minutes. Initial experiments were conducted in samples prepared from citrated blood, although lepirudinised samples were also included in later experiments.

As the results from Chapter 4 indicated little difference between ticagrelor and PAM *in vitro* the choice of $P2Y_{12}$ receptor blocker was not deemed critical in these experiments. In order to relate to the clinical study data, with prasugrel, PAM was chosen to model strong $P2Y_{12}$ receptor blockade. As such, 3μM PAM was used as a treatment.

6.2 Methods

6.2.1 Preparation of platelet solutions

PRP and PPP were prepared from whole blood anticoagulated with either citrate or lepirudin, as described in sections 2.3 and 2.4.

6.2.2 Incubation of platelet-rich plasma

Samples of PRP were pre-incubated for 30 min at 37°C with PAM (3µM in 0.05% DMSO), and/or aspirin (30µM), or vehicle (0.05% DMSO) before being rested at room temperature prior to use in light transmission aggregometry.

6.2.3 Determination of platelet aggregation by traditional LTA

In all studies a PAP-8E aggregometer was used (BioData Corporation, USA). Before each measurement cycle a blank reference reading was made using a 250µl sample of PPP from the relevant donor. To follow aggregation responses, 225µl samples of pre-incubated PRP were placed into individual cuvettes containing siliconised magnetic stir bars (Alpha Laboratories, UK) and incubated for 90 s at 37°C with continuous stirring at either 100, 300 or 1200rpm. 25µl aliquots of individual platelet agonists (at 10 times final concentration) were then added and aggregation responses followed with continuous stirring at the same speed as during the pre-incubation period. The percentage aggregation within each cuvette was calculated internally by the instrument, using the absorbance of the blank PPP as a surrogate for 100% aggregation.

6.2.4 TXB_2 assay

At the end of the PRP aggregation monitoring, cyclo-oxygenase activity was halted by the addition of 1mM diclofenac, the samples were transferred from the

aggregometer cuvette to a 96-well microtitre plate and centrifuged at 1300 x g for 10 minutes at 5°C, and the supernatants removed and frozen. TXB_2 was determined using either the overnight assay kit (5 minute experiments) [section 2.8.2], or the HTRF assay kit (20 minute experiments) [section 2.8.3].

6.3 Results

6.3.1 Characterisation of ADP, collagen and U46619-induced responses in traditional LTA

Pilot experiments were conducted to characterise the aggregatory responses in traditional LTA to ADP, U46619 and collagen. A range of concentrations were used and the experiments were all performed using the default stir-speed setting of the aggregometer, 1200rpm.

Similarly to 96-well plate aggregometry, traditional LTA is a sensitive tool to assess ADP-induced platelet aggregation. At low concentrations of ADP an initial aggregation-type response progresses to disaggregation, with a low final aggregation. At higher concentrations, the initial response is stabilised at five minutes. Aspirin had only a very weak inhibitory effect against ADP-induced aggregation in traditional LTA [figure 6.1]. In the presence of PAM, a small initial response, consistent with the actions of ADP on $P2Y_1$ receptors was seen in the presence of 3µM PAM, although $P2Y_{12}$ receptor blockade strongly inhibited responses to ADP [figure 6.1].

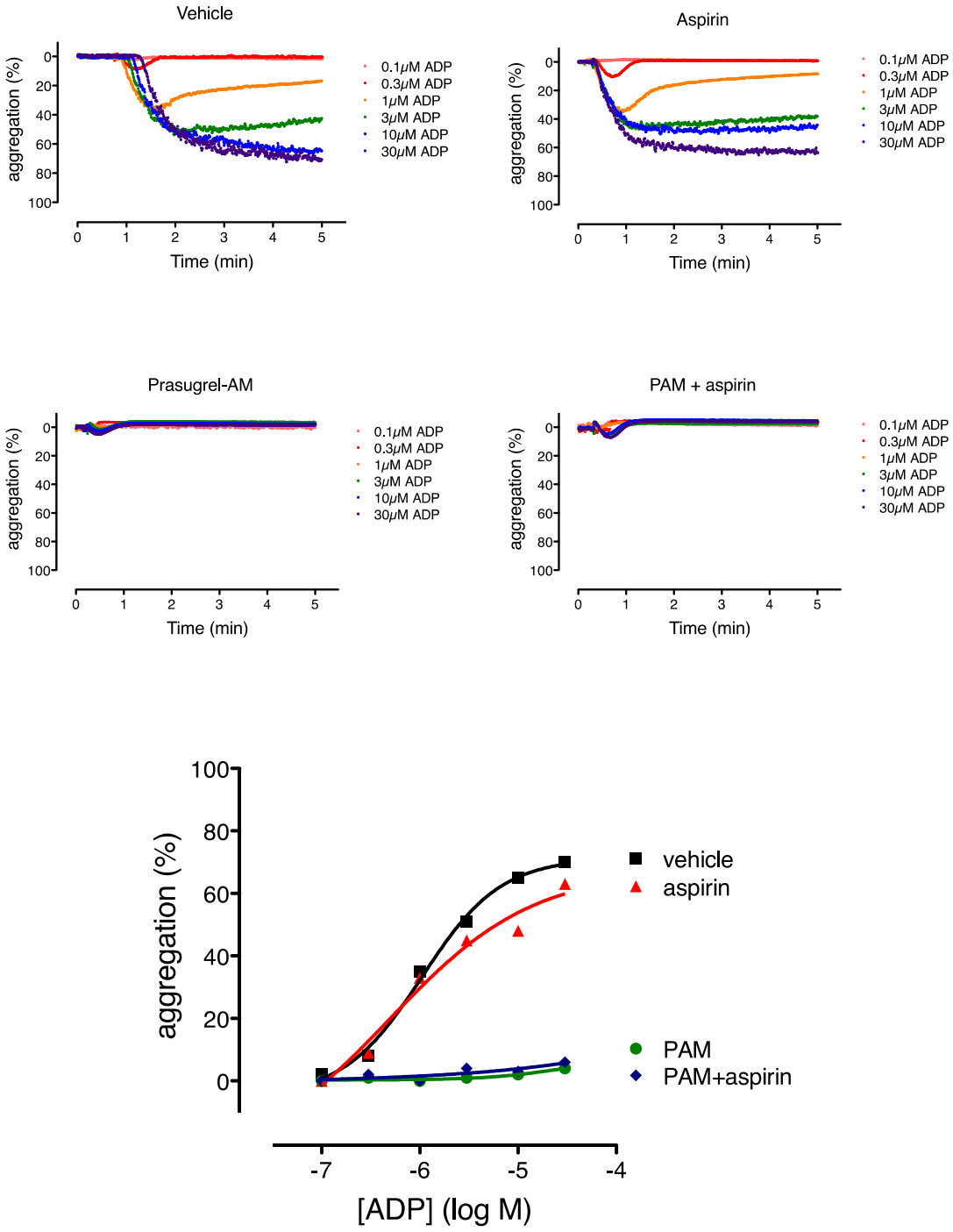


Figure 6.1 ADP induced aggregation in traditional LTA.

Aggregation time-response traces (upper four panels) and concentration-response curves at 5min (lower panel) to ADP (0.1-30μM) in the presence of aspirin (30μM), PAM (3μM), PAM+aspirin or vehicle (0.1% DMSO) measured by traditional LTA in citrated PRP. Pilot data from a single donor.

Figure 6.2 shows U46619-induced platelet aggregation is little affected by 30µM aspirin alone in traditional LTA, although 3µM PAM strongly inhibits responses at lower concentrations of U46619 with a moderate effect against the highest concentrations used. In this pilot study, aspirin did have a small additional effect when used with PAM, suggesting that TXA_2 production contributes to the aggregation response.

Collagen induced strong aggregatory responses under control conditions, even at low concentrations [figure 6.3]. The inhibition of these responses by either PAM or aspirin alone was similar; the inhibition of responses was highest at low concentrations of collagen and limited at the highest concentrations. As expected, collagen-induced aggregation was strongly inhibited in the presence of PAM plus aspirin [figure 6.3].

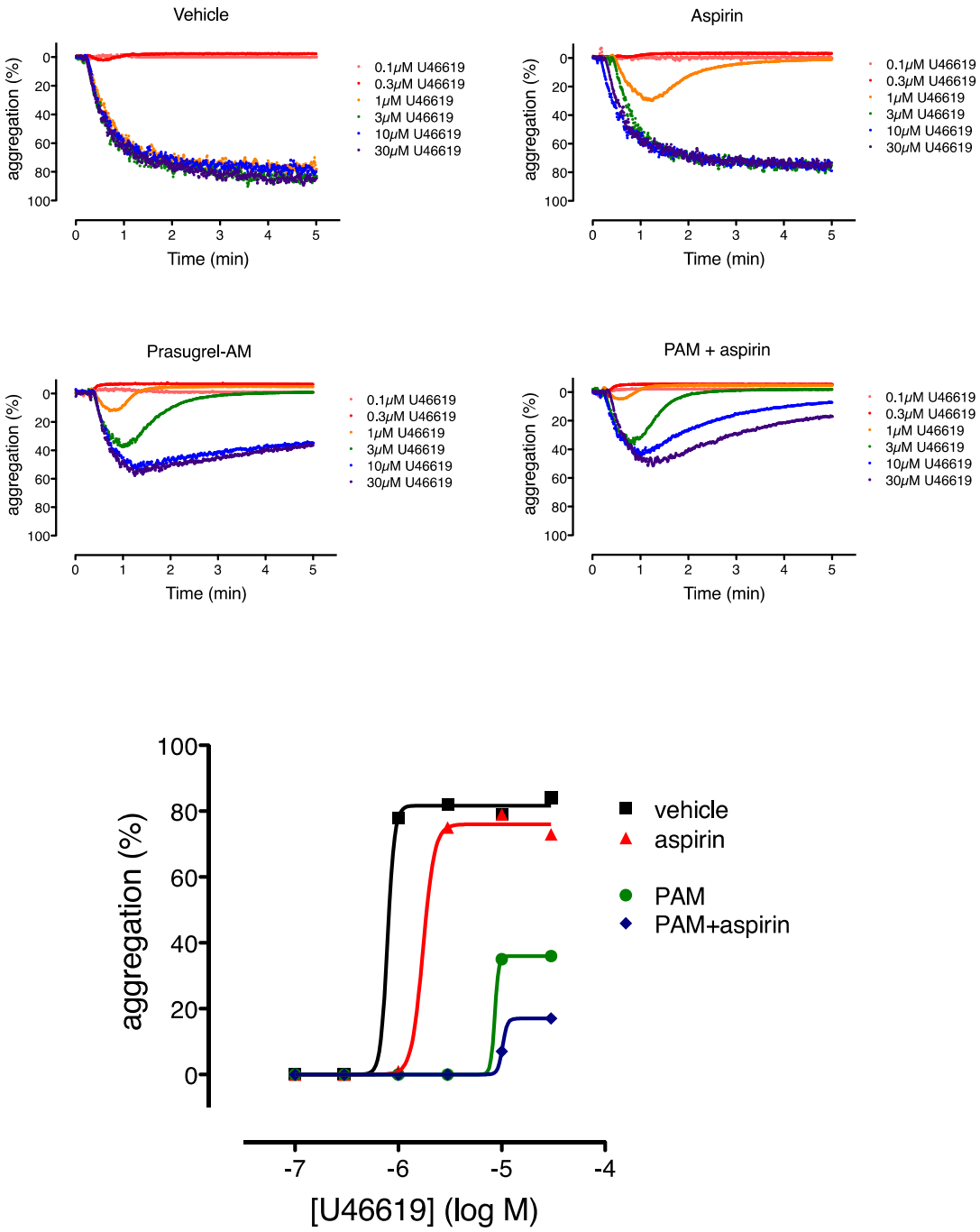


Figure 6.2 U46619 induced aggregation in traditional LTA.

Aggregation time-response traces (upper four panels) and concentration-response curves at 5min (lower panel) to U46619 (0.1-30μM) in the presence of aspirin (30μM), PAM (3μM), PAM+aspirin or vehicle (0.1% DMSO) measured by traditional LTA in citrated PRP. Pilot data from a single donor.

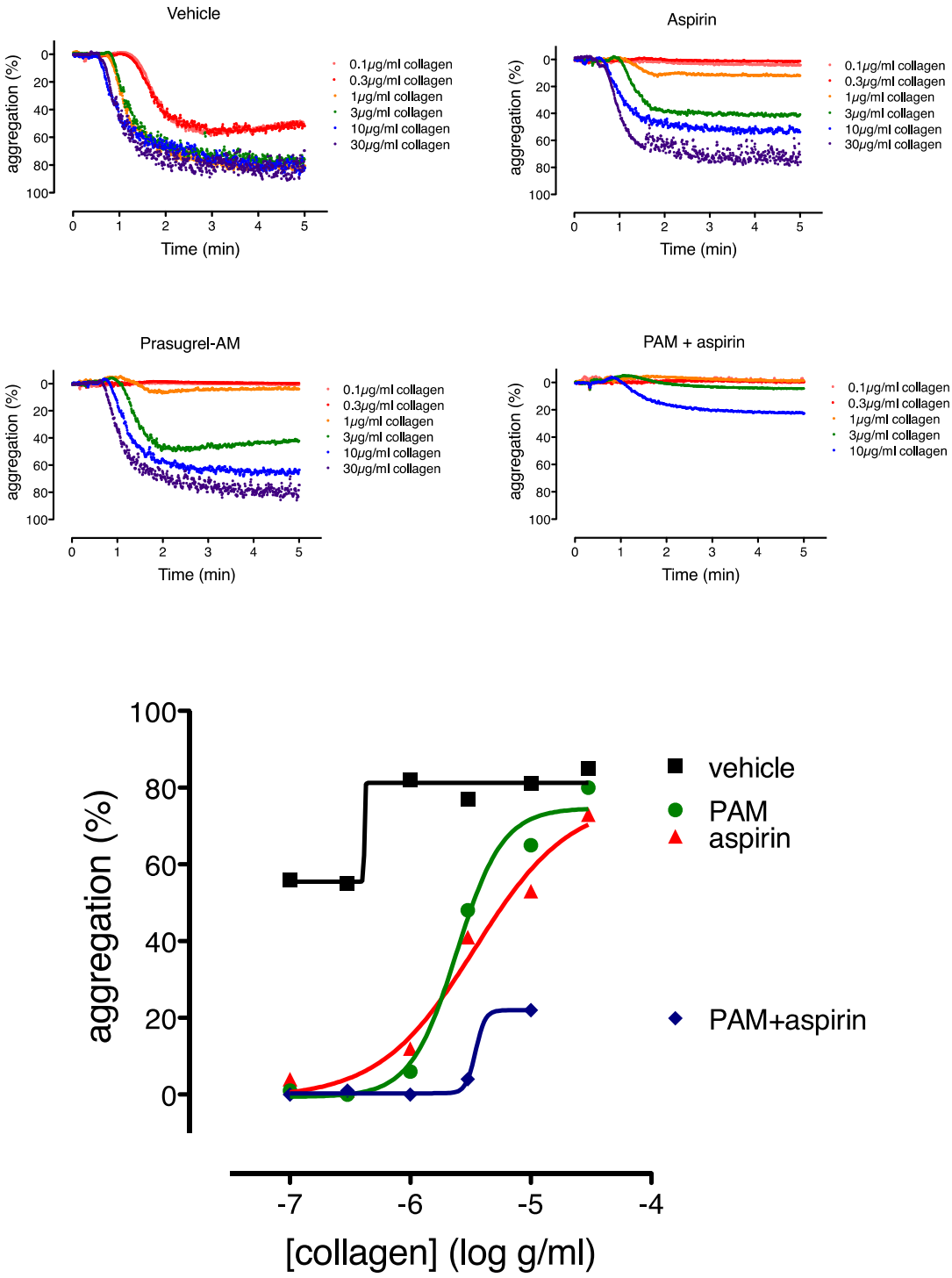


Figure 6.3 Collagen induced aggregation in traditional LTA.

Aggregation time-response traces (upper four panels) and concentration-response curves at 5min (lower panel) to collagen (0.1-30µg/ml) in the presence of aspirin (30µM), PAM (3µM), PAM+aspirin or vehicle (0.1% DMSO) measured by traditional LTA in citrated PRP. Pilot data from a single donor.

6.3.2 The effect of stir-speed on collagen-induced aggregation and TXA_2 production

To investigate the effect of stir-speed on collagen-induced aggregation responses, in the absence of any platelet antagonists, aggregation studies were performed at 100rpm, 300rpm and 1200rpm against a range of collagen concentrations (0.01-30µg/ml). As shown in figure 6.4, increasing the stir-speed caused a leftward shift in the collagen concentration-response curve, indicating an increased sensitivity to collagen at higher stir-speeds. This change in sensitivity was approximately one order of magnitude between aggregation performed at 100rpm and 1200rpm. Despite this, the maximum collagen-induced response was equivalent across all stir-speeds [figure 6.4]. Having characterised the concentration-dependent response of collagen-induced aggregation a single concentration was selected to study the effects of platelet antagonists on collagen-induced responses at different stir speeds. The criterion of this selection was the lowest concentration to cause a maximal response at all stir speeds, which was 3µg/ml [figure 6.4].

Although the maximum aggregation to 3µg/ml collagen did not change with speed in untreated samples, in the presence of platelet antagonists there was a large difference in responses. At either 100rpm or 300rpm aspirin inhibited collagen-induced aggregation by approximately 80-95%, although at 1200rpm, the inhibition of aggregation was less than 60%. Similarly, PAM had a stronger effect at 100rpm, inhibiting aggregation by 70%, although at 300rpm, this was reduced to only 30% inhibition, with less than 20% inhibition at 1200rpm. However, when aspirin and PAM were used in combination a near maximal inhibitory effect was achieved, that was only slightly reduced at 1200rpm [figure 6.5].

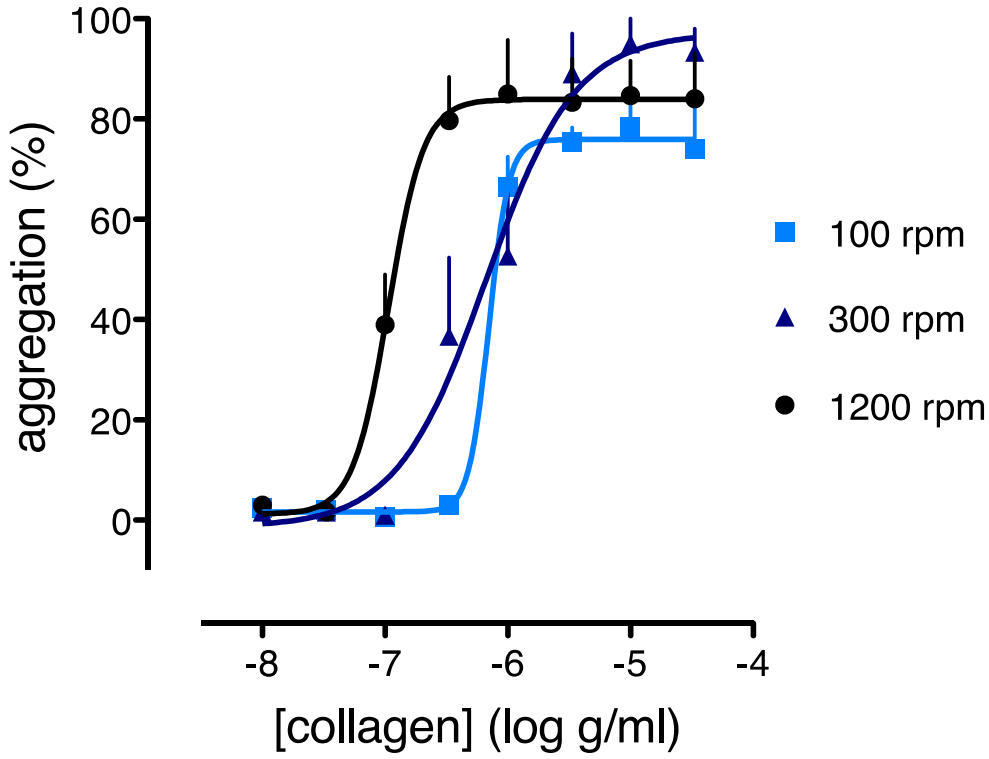


Figure 6.4 The effect of stir-speed on collagen induced platelet aggregation.
Concentration response curves to collagen (0.01-30µg/ml) at stir-speeds of 100, 300 and 1200 rpm in traditional LTA for 5min. Experiments were conducted in citrated PRP. Each point represents the mean response from 3 donors and vertical bars indicate standard error of the mean.

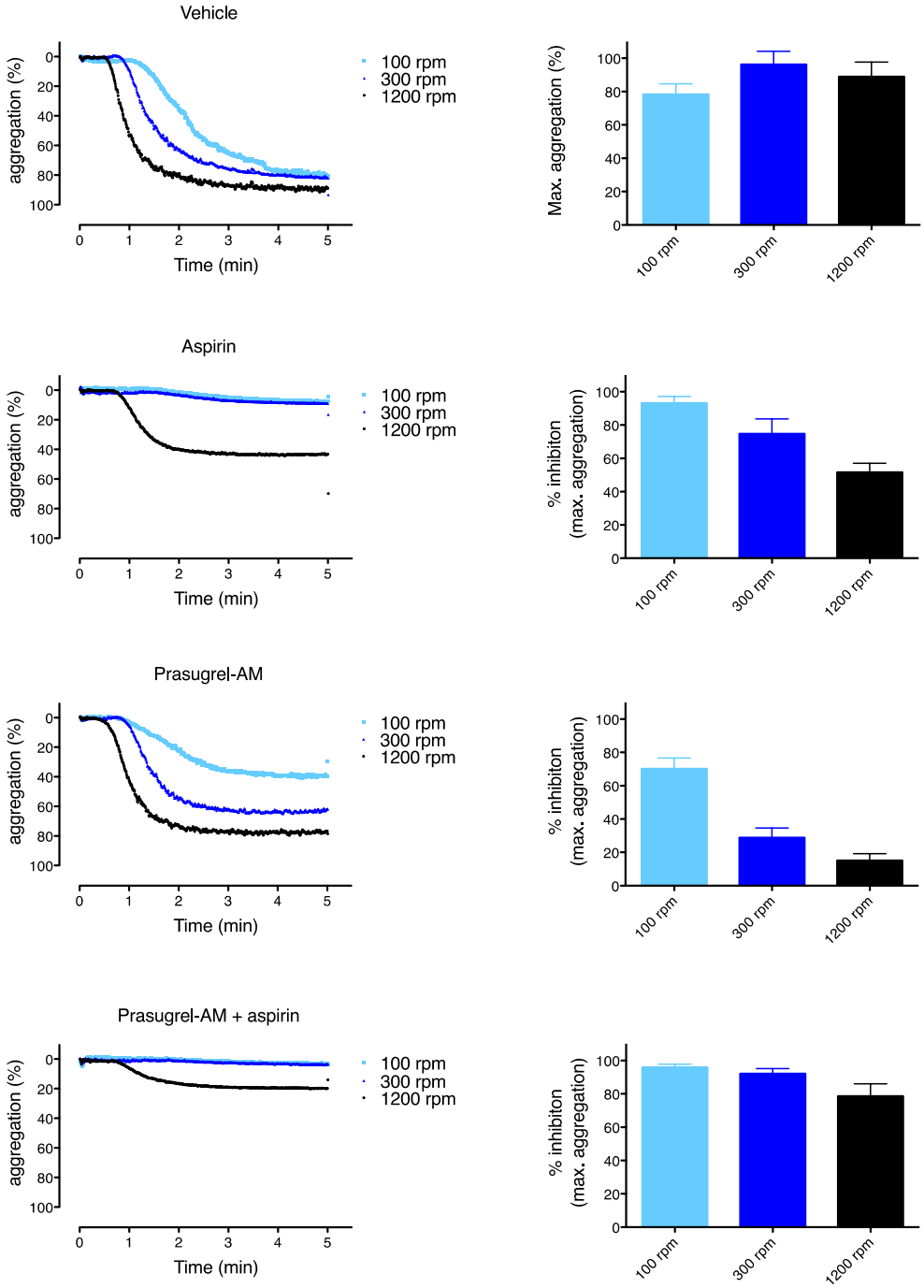


Figure 6.5 The effects of aspirin and PAM on collagen induced platelet aggregation at 100, 300 and 1200rpm.

Left hand panels, aggregation time-response traces to 3µg/ml collagen at 100, 300 and 1200rpm. Each line represents the mean response from 4 individuals; Right hand panels, column charts showing either maximum aggregation (vehicle, 0.1% DMSO) or percent inhibition of maximum aggregation to 3µg/ml collagen. Data are mean ± s.e.m. from 4 individuals. Responses were measured by traditional LTA in citrated PRP treated with aspirin (30µM), PAM (3µM), PAM+aspirin or vehicle.

Figure 6.6 shows the additional inhibitory effect of aspirin to PAM for collagen-induced aggregation by traditional LTA. At low stir-speeds, aspirin has a small additional effect to PAM, although at higher stir-speeds PAM is less effective and so the effect of aspirin is amplified. To further investigate the mechanism of this effect, TXA_2 production was measured under each condition. As shown in figure 6.7, TXA_2 production was substantially higher at 300rpm and 1200rpm, than at 100rpm. Whilst the presence of aspirin abolished TXA_2 production at any speed, PAM had little effect at 100rpm, 300rpm or 1200rpm. These results contrasted with the TXA_2 production during 96-well plate aggregometry, which was lower than production at even 100rpm in traditional LTA.

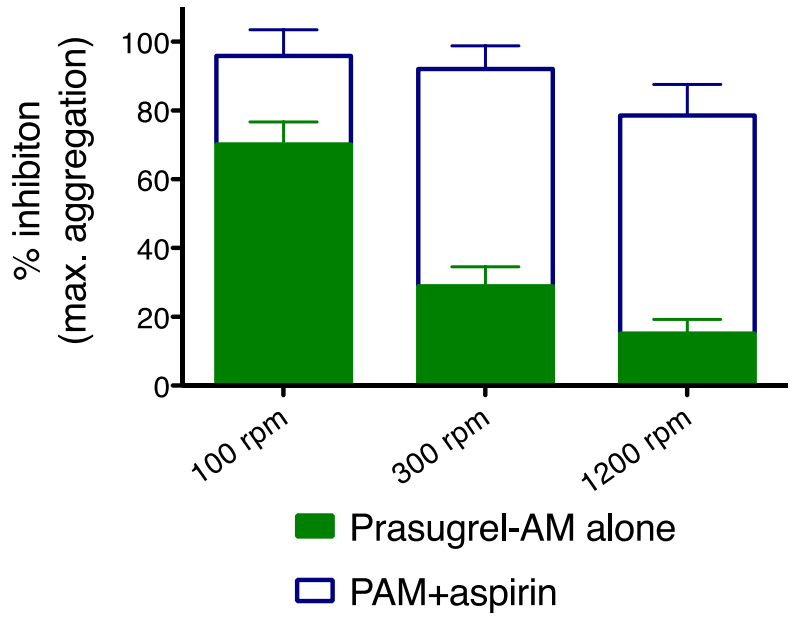


Figure 6.6 Additional effects of aspirin in the presence of PAM at 100, 300 and 1200rpm.

Column charts, constructed from data in figure 6.5, to illustrate the additional effects of aspirin against aggregation induced by 3µg/ml collagen in the presence of PAM at 100, 300 and 1200rpm. Data are mean ± s.e.m. from 4 individuals. Responses were measured by traditional LTA in citrated PRP treated with PAM (3µM), PAM and aspirin (30µM).

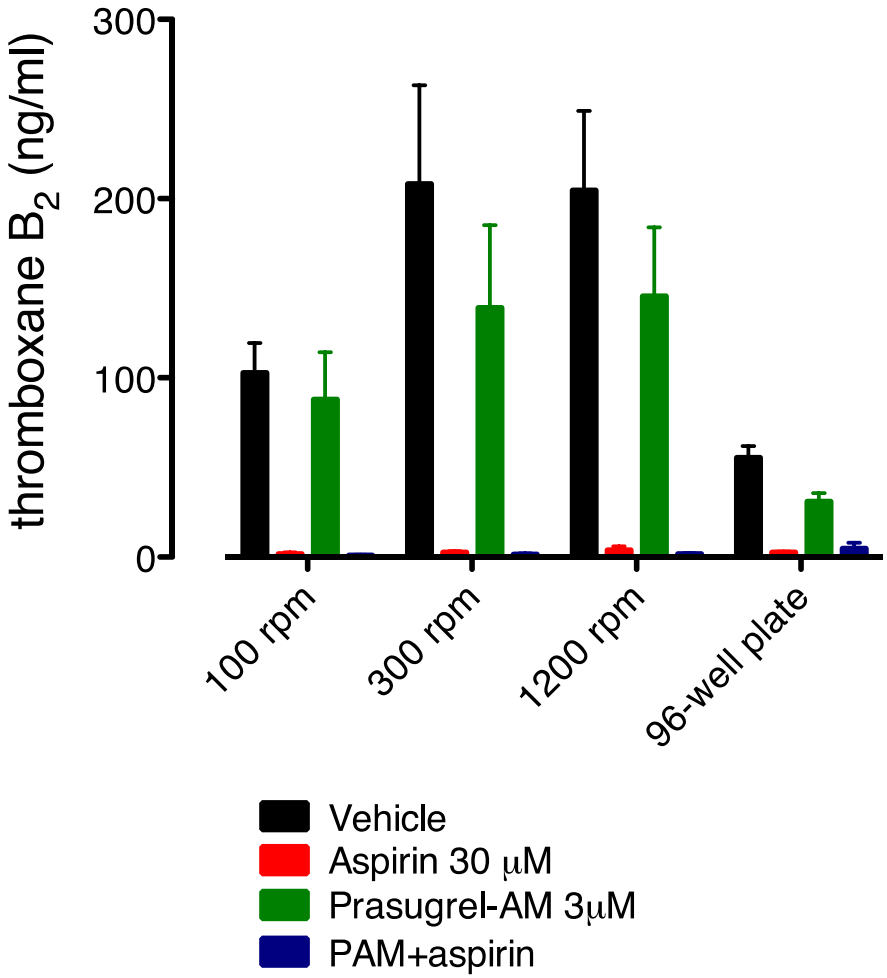


Figure 6.7 TXB_2 production during collagen induced platelet aggregation by traditional and 96-well plate LTA.

TXB_2 production during aggregation to 3μg/ml collagen by either traditional LTA, performed at 100, 300 or 1200rpm, or 96-well plate aggregometry. Experiments were performed in citrated PRP treated with either vehicle (0.1% DMSO), aspirin (30μM), PAM (3μM) or PAM+aspirin. Data are mean ± s.e.m. for 4 individuals.

6.3.3 The effect of stir-speed on U46619-induced aggregation

As TXA_2 production was increased with stir-speed, TXA_2 signaling was investigated using the stable thromboxane mimetic U46619. Pilot data, shown in figure 6.2, indicated that 3µM U46619 is the lowest concentration to induce a maximal response at 1200rpm and so this concentration was used in experiments to compare the effects of aspirin and PAM at 100, 300 and 1200rpm.

Whilst 3µM U46619-inudced aggregation tended to be higher at either 300rpm or 1200rpm than at 100rpm these changes were not statistically significant. Aspirin did not appreciably affect U46619-induced aggregation at any speed, either alone or when combined with PAM, although PAM inhibited U46619-induced aggregation in a speed-dependent manner, with the greatest inhibition at 100rpm [figure 6.8]. There was no further reduction in aggregation responses in the presence of aspirin.

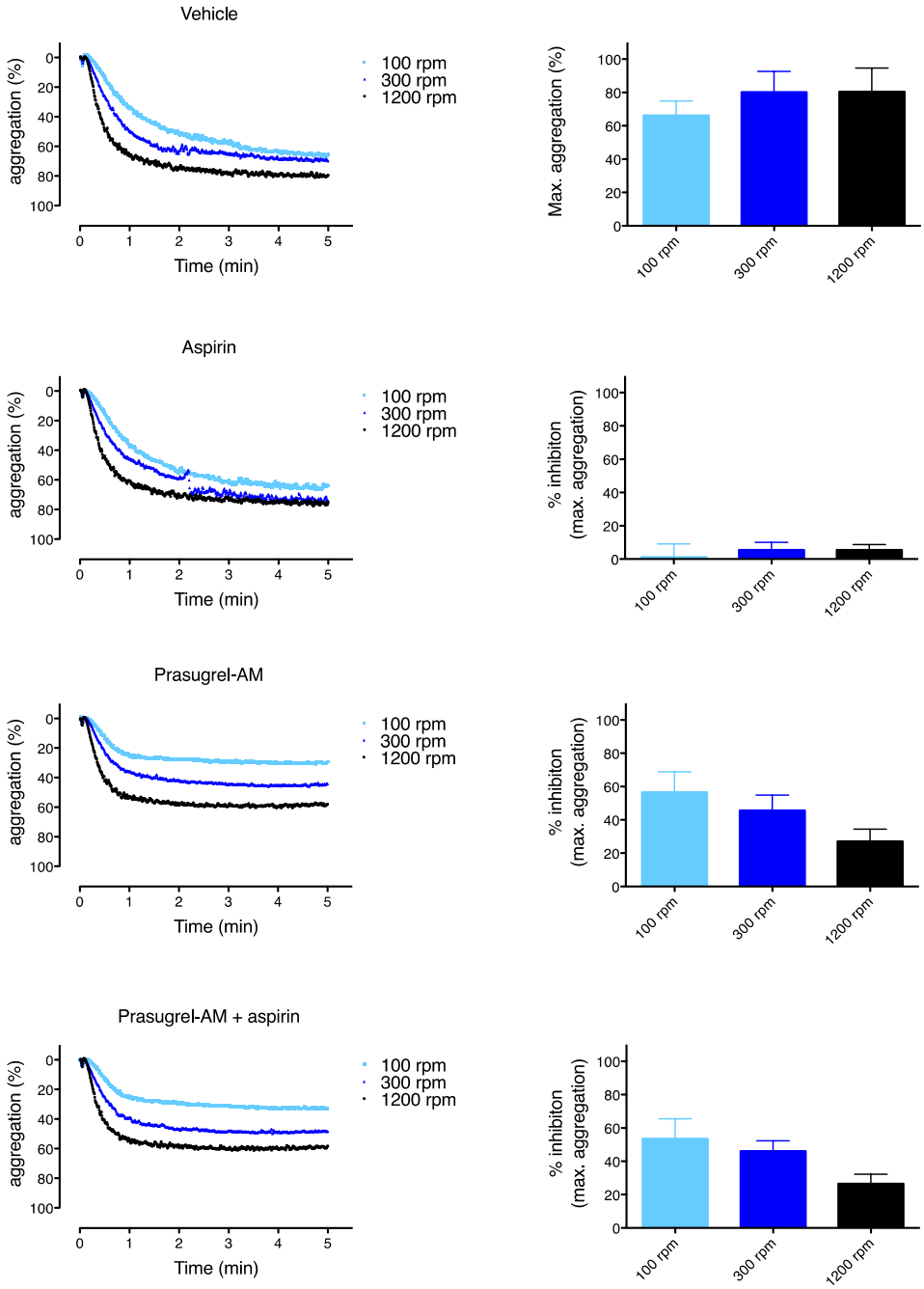


Figure 6.8 The effects of aspirin and PAM on U46619 induced platelet aggregation at 100, 300 and 1200rpm.

Left hand panels, aggregation time-response traces to 3µM U46619 at 100, 300 and 1200rpm. Each line represents the mean response from 4 individuals; right hand panels, column charts showing either maximum aggregation (vehicle, 0.1% DMSO) or percent inhibition of maximum aggregation to 3µM U46619. Data are mean ± s.e.m. from 4 individuals. Responses were measured by traditional LTA in citrated PRP treated with aspirin (30µM), PAM (3µM), PAM+aspirin or vehicle.

6.3.4 The effect of stir-speed on collagen-related peptide (CRP-XL)-induced platelet aggregation

To further understand the effects of collagen-induced aggregation, which occurs in response to concerted GPVI and integrin alpha2ß1 signaling, the GPVI-specific agonist collagen-related peptide was used to induce platelet aggregation in the presence of platelet antagonists across a range of speeds.

In keeping with other agonists, the maximum response to 100 ng/ml CRP-XL was little affected by speed, although there was a very small trend to increased aggregation at 300rpm and 1200rpm compared to 100rpm [figure 6.9]. Although the magnitude of aggregation measurements varied, the percentage inhibition achieved with 30µM aspirin was similar at each stir-speed, and was close to 50%. PAM had a similar, although slightly stronger, effect to aspirin, inhibiting aggregation by approximately 60% at 100rpm and 300rpm, and by approximately 50% at 1200rpm. When used in combination, PAM and aspirin had an additive effect, inhibiting CRP-XL-induced responses by more than 80%.

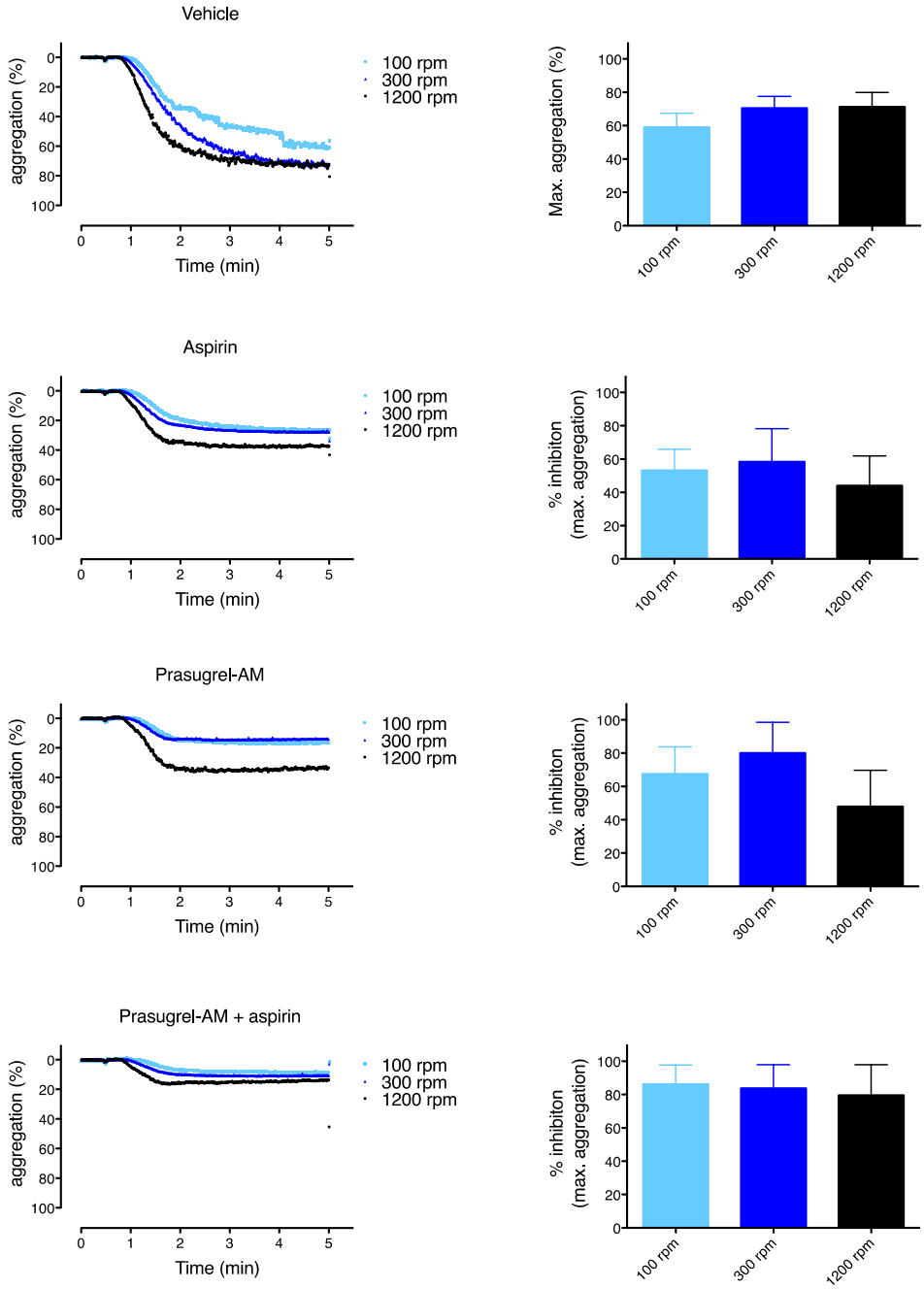


Figure 6.9 The effects of aspirin and PAM on CRP-XL induced platelet aggregation at 100, 300 and 1200rpm.

Left hand panels, aggregation time-response traces to 100ng/ml CRP-XL at 100, 300 and 1200rpm. Each line represents the mean response from 4 individuals; right hand panels, column charts showing either maximum aggregation (vehicle, 0.1% DMSO) or percent inhibition of maximum aggregation to 100ng/ml CRP-XL. Data are mean ± s.e.m. from 4 individuals. Responses were measured by traditional LTA in citrated PRP treated with aspirin (30µM), PAM (3µM), PAM+aspirin or vehicle.

6.3.5 The effect of anticoagulant, stir speed and time on platelet aggregation responses in traditional LTA

Stir-speed appears to be an important factor in platelet responses modeled by traditional LTA. Changes in stir-speed may be associated with changes in TXA_2 production, which in turn affects the sensitivity of this assay to platelet antagonists. These experiments used PRP from citrated blood, which, as explained in chapter 3, may promote TXA_2 production. Therefore, the effect of stir-speed should also be considered in PRP from lepirudinised blood. The experiments described above were all conducted for only 5 minutes, by which point responses had generally stabilised at a maximum. However, it is unclear whether or not responses change if traditional LTA is performed over longer periods of time. Investigation of time effects in traditional LTA may help to explain the differences between this method and 96-well plate aggregometry.

Considering these points, the effect of stir-speed on platelet aggregation induced by a range of agonists was studied, over a period of 20 minutes using PRP prepared from both citrated and lepirudinised whole blood.

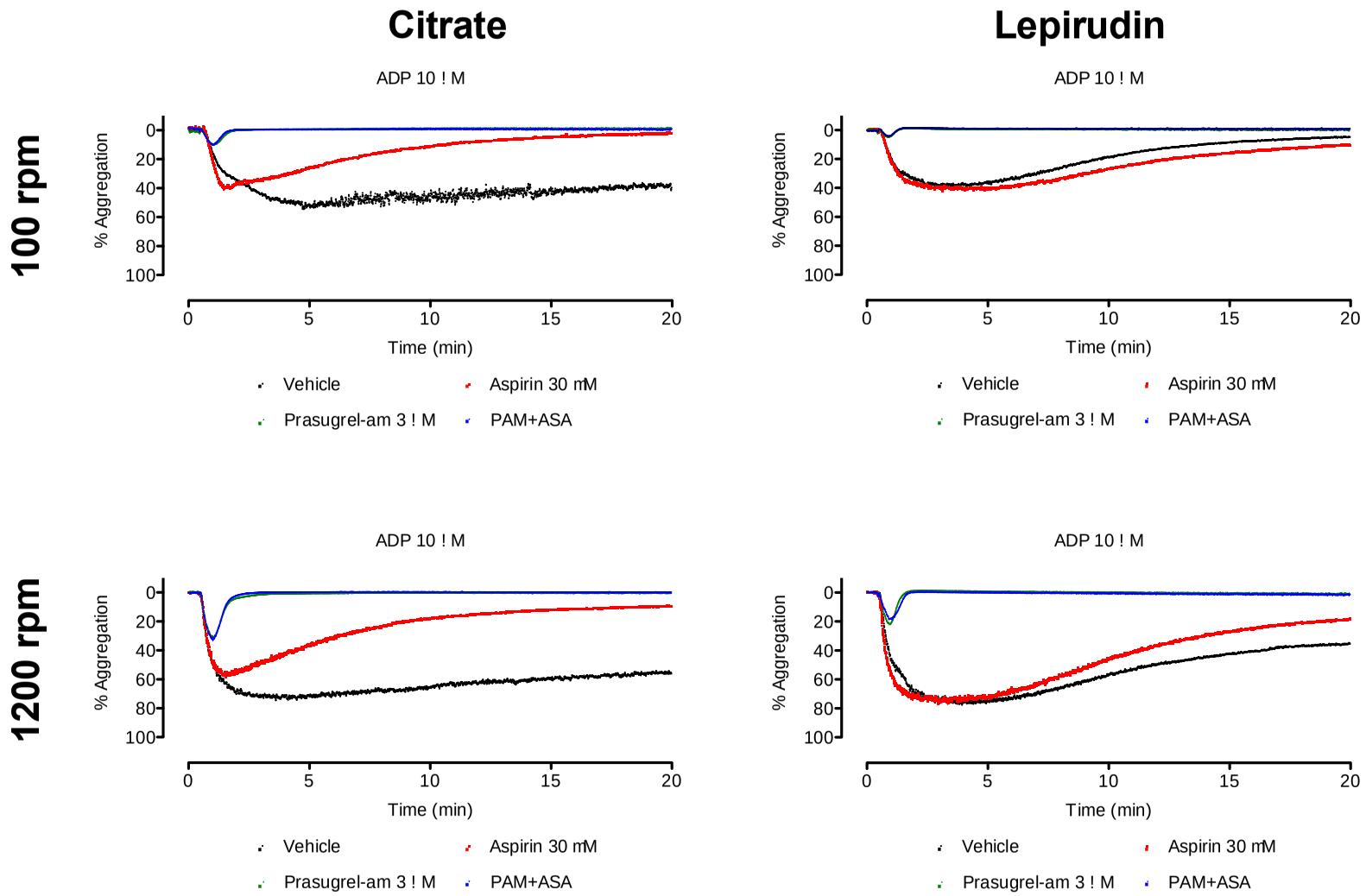
6.3.5.1 ADP

In citrated PRP, ADP-induced aggregation was generally stable in the absence of platelet antagonists and there was a slightly higher aggregation response at 1200rpm. At either speed, aspirin treated samples showed a similar primary response to untreated samples, although this was not sustained and disaggregation occurred over the course of the assay, with partial disaggregation by 5 minutes and near complete disaggregation at 20 minutes [figure 6.10]. Responses in lepirudin treated samples were quite different. Initial aggregation responses to ADP were greater at 1200rpm than 100rpm and these responses, which were sustained until 5

minutes, were similar in both vehicle and aspirin treated samples. Despite this initial strong response, in both treatments, near-complete disaggregation occurred by 20 minutes at 100rpm, and a similar but less complete disaggregation occurred in samples stirred at 1200rpm. PAM fully inhibited ADP-induced aggregation, although in both citrated and lepirudinised samples reversible aggregations, attributed to $P2Y_1$ receptor activation, were seen. These reversible responses were enhanced by higher speeds and were greater in citrated samples [figure 6.10].

Figure 6.10 (opposite page) ADP induced aggregation in citrate and lepirudin at low and high speeds.

Aggregation traces showing the effects of time, stir-speed (100 or 1200rpm) and anticoagulant (citrate or lepirudin) on platelet aggregation in response to 10µM ADP as measured by traditional LTA. PRP samples were treated with either vehicle (0.1% DMSO), aspirin (30µM), PAM (3µM) or PAM+aspirin. Agonist was added 30 seconds after the start of the assay, which ran for 20 minutes. Data represent the mean response of 4 individuals.


Citrate
Lepirudin
100 rpm
1200 rpm
ADP 10 ! M
% Aggregation
0
20
40
60
80
100
0
5
10
15
20
Time (min)
Vehicle
Aspirin 30 mM
Prasugrel-am 3 ! M
PAM+ASA

6.3.5.2 TRAP-6 amide

In citrated treated PRP, TRAP-6 amide induced aggregation was stable until 20 minutes, although in the presence of aspirin this stability was reduced. Despite this, at 5 minutes aspirin appeared to have a relatively small effect against aggregations at either 100rpm or 1200rpm. The primary response to TRAP-6 amide was enhanced with speed in the presence of PAM, or PAM+aspirin, and similarly to aspirin, little inhibition was seen at 5 minutes, although by 20 minutes much of the aggregatory response had reversed [figure 6.11].

At 20 minutes, the effect of PAM alone was very similar to the effect of PAM in the presence of aspirin. In lepirudin, aggregation was less stable, with some disaggregation by 20 minutes, although aspirin had little effect on these responses. The effect of PAM was considerable; a strong primary response was rapidly reversed, and by 20 minutes this reversal was complete [figure 6.11].

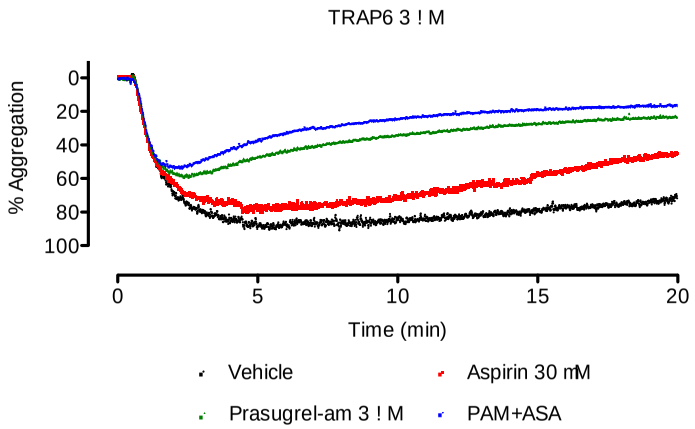
Figure 6.11 (opposite page) TRAP-6 amide induced aggregation in citrate and lepirudin at low and high speeds.

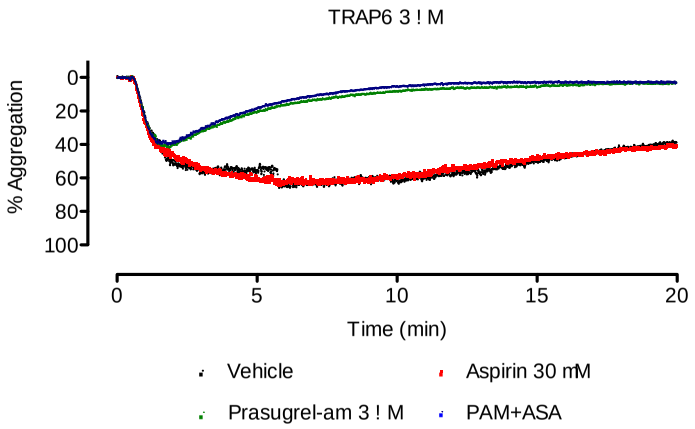
Aggregation traces showing the effects of time, stir-speed (100 or 1200rpm) and anticoagulant (citrate or lepirudin) on platelet aggregation induced by 3µM TRAP-6 as determined by traditional LTA. PRP samples were treated with either vehicle (0.1% DMSO), aspirin (30µM), PAM (3µM) or PAM+aspirin. Agonist was added 30 seconds after the start of the assay, which ran for 20 minutes. Data represent the mean response of 4 individuals.

Citrate

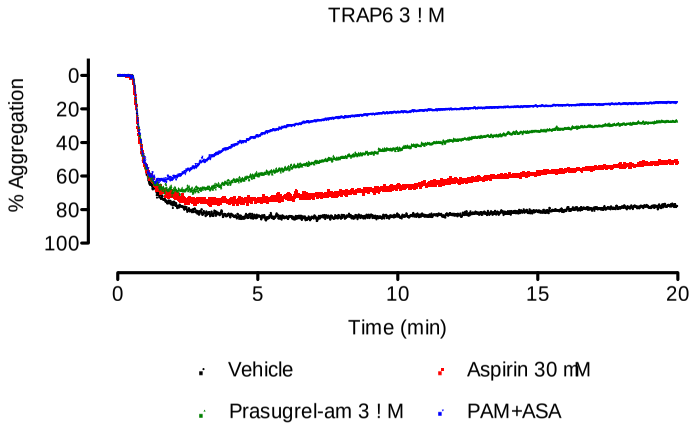
Lepirudin

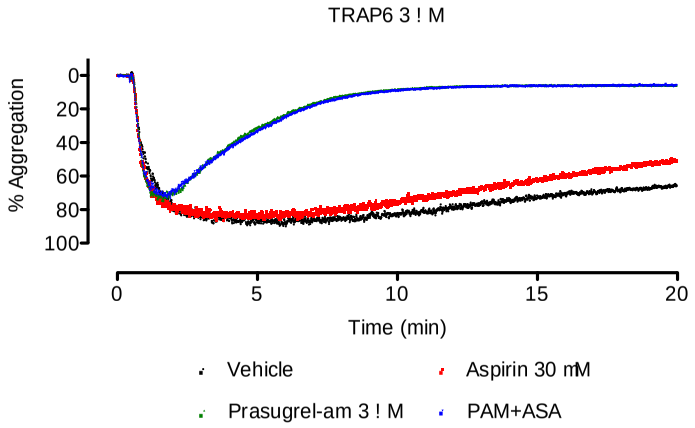
100 rpm





1200 rpm





6.3.5.3 Collagen and CRP-XL

Collagen induced a large and sustained aggregation response in PRP treated with either citrate or lepirudin at 1200rpm, although at 100rpm the final aggregation was slightly lower in lepirudinised, although not citrated samples [figure 6.12 and table 6.1]. Aspirin and PAM were both more effective against aggregations at 100rpm, than at 1200rpm in citrated samples and whilst there was some disaggregation with both antagonists, this was more marked in the case of PAM [figure 6.12 and table 6.1]. In lepirudinised samples, both PAM and aspirin (individually) had only a weak effect at 5 minutes, although by 20 minutes, this inhibitory effect was enhanced due to disaggregation. This effect was more pronounced at 100rpm than at 1200rpm. Aspirin and PAM used together had a strong inhibitory effect under all experimental conditions [figure 6.12 and table 6.1].

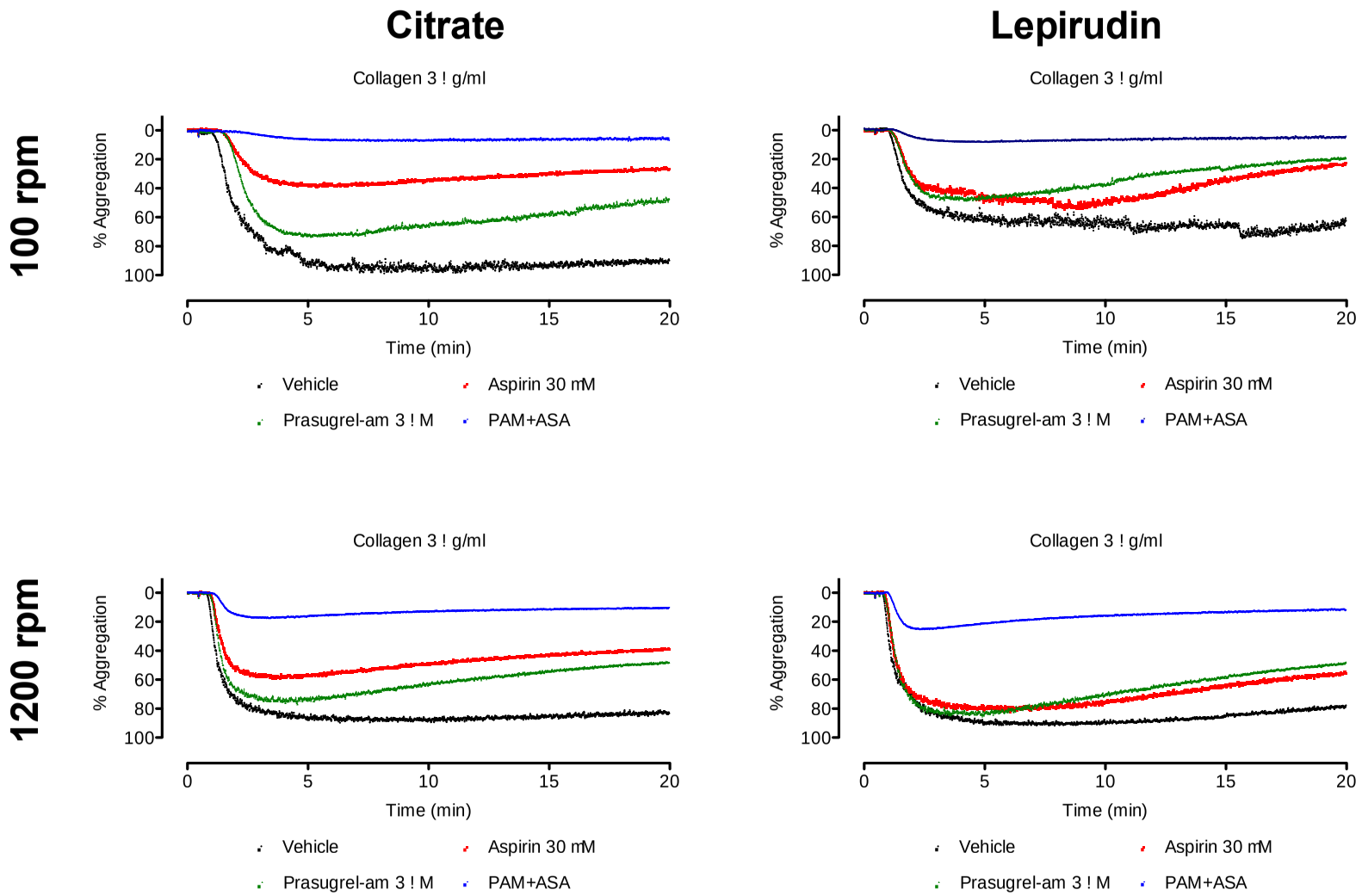
Collagen 3µg/ml		Citrate		Lepirudin	
		100 rpm	1200 rpm	100 rpm	1200 rpm
Aspirin 30µM % inhibition	5 minutes	58±9%	34±3%	26±6%	10±3%
	20 minutes	68±8%	52±2%	64±4%	27±9%
PAM 3µM % inhibition	5 minutes	22±3%	14±3%	16±17%	8±6%
	20 minutes	45±4%	41±4%	69±3%	39±11%
PAM+Aspirin % inhibition	5 minutes	92±1%	82±2%	87±1%	76±4%
	20 minutes	93±1%	87±2%	92±1%	83±4%

Table 6.1 Percent inhibition of collagen induced aggregation

Numerical summary of the aggregation data shown in figure 6.12

Figure 6.12 (opposite page) Collagen induced aggregation in citrate and lepirudin at low and high speeds.

Aggregation traces showing the effects of time, stir-speed (100 or 1200rpm) and anticoagulant (citrate or lepirudin) on platelet aggregation induced by 3µg/ml collagen determined by traditional LTA. PRP samples were treated with either vehicle (0.1% DMSO), aspirin (30µM), PAM (3µM) or PAM+aspirin. Agonist was added 30 seconds after the start of the assay, which ran for 20 minutes. Data represent the mean response of 4 individuals.


Citrate
Lepirudin
100 rpm
1200 rpm
Collagen 3 ! g/ml
% Aggregation
0
20
40
60
80
100
0
5
10
15
20
Time (min)
Vehicle
Aspirin 30 mM
Prasugrel-am 3 ! M
PAM+ASA

Aggregation induced by CRP-XL-induced was slightly enhanced at 1200rpm in relation to 100rpm, and responses were greater in citrated than in lepirudinised samples. Some disaggregation occurred in control samples, and the magnitude of this was greater in the presence of lepirudin than in citrate [table 6.2]. The sensitivity of CRP-XL-induced aggregation to PAM was little affected by speed and whilst PAM had a strong effect in citrate, the effect in lepirudin was stronger, with near total inhibition of aggregation responses [figure 6.13 and table 6.2]. In contrast, aspirin had no effect on aggregation at 5 minutes and only a small effect by 20 minutes in lepirudinised samples, although its effects in citrate were near identical to those of PAM [figure 6.13 and table 6.2]. In citrate, although not in lepirudin, PAM and aspirin furthered the inhibitory effect of either of these drugs used alone, completely inhibiting aggregation [figure 6.13 and table 6.2].

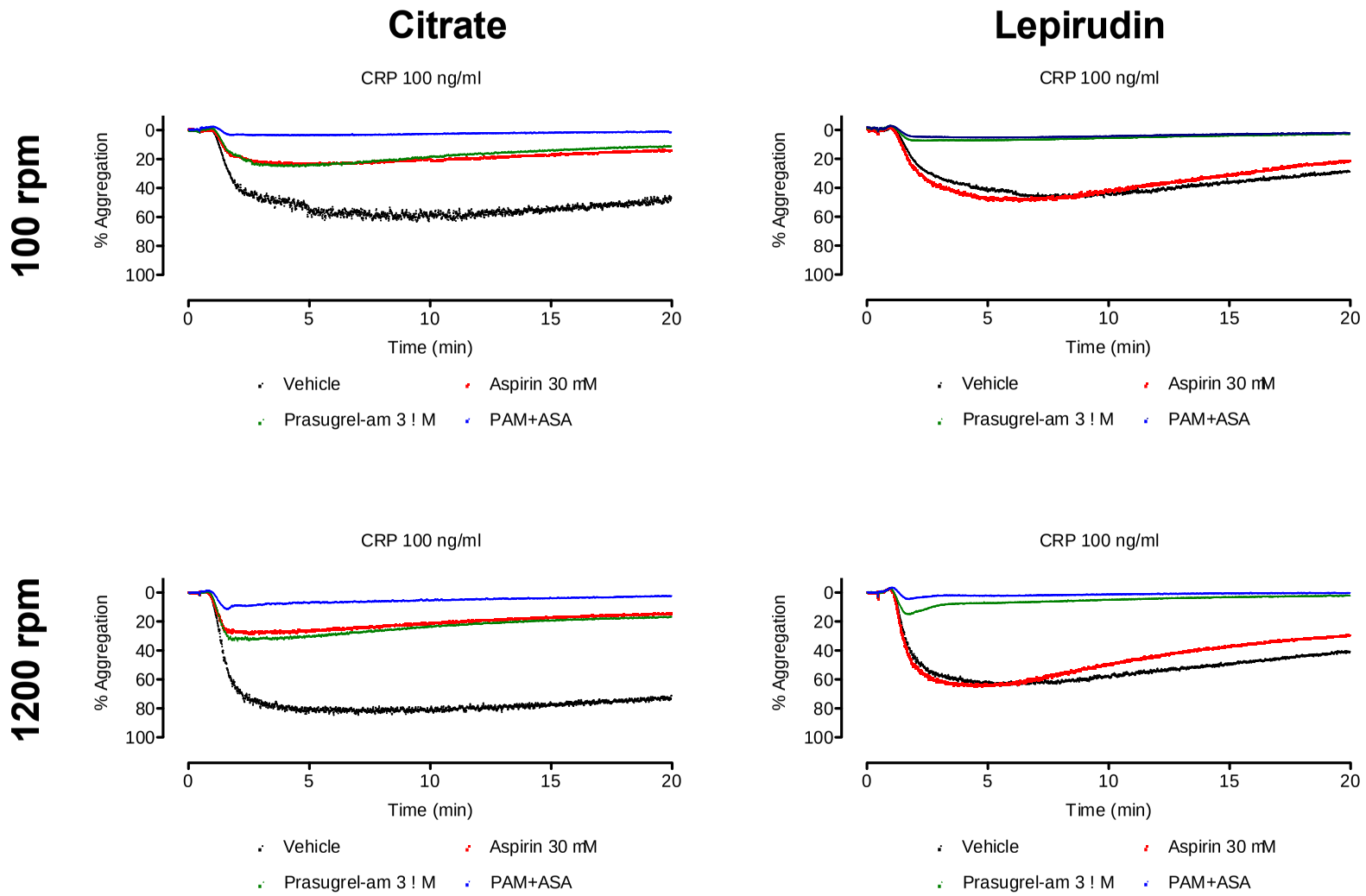
CRP-XL 100ng/ml		Citrate		Lepirudin	
		100 rpm	1200 rpm	100 rpm	1200 rpm
Aspirin 30µM % inhibition	5 minutes	63±11%	62±18%	-8±13%	5±21%
	20 minutes	71±9%	77±11%	17±8%	17±28%
PAM 3µM % inhibition	5 minutes	66±14%	58±23%	87±6%	90±1%
	20 minutes	82±8%	75±15%	92±4%	96±2%
PAM+Aspirin % inhibition	5 minutes	93±5%	89±8%	90±5%	97±2%
	20 minutes	96±3%	96±2%	91±4%	96±3%

Table 6.2 Percent inhibition of CRP-XL induced aggregation

Numerical summary of the aggregation data shown in figure 6.13

Figure 6.13 (opposite page) CRP-XL induced aggregation in citrate and lepirudin at low and high speeds.

Aggregation traces showing the effects of time, stir-speed (100 or 1200rpm) and anticoagulant (citrate or lepirudin) on platelet aggregation induced by 100ng/ml CRP-XL determined by traditional LTA. PRP samples were treated with either vehicle (0.1% DMSO), aspirin (30µM), PAM (3µM) or PAM+aspirin. Agonist was added 30 seconds after the start of the assay, which ran for 20 minutes. Data represent the mean response of 4 individuals.

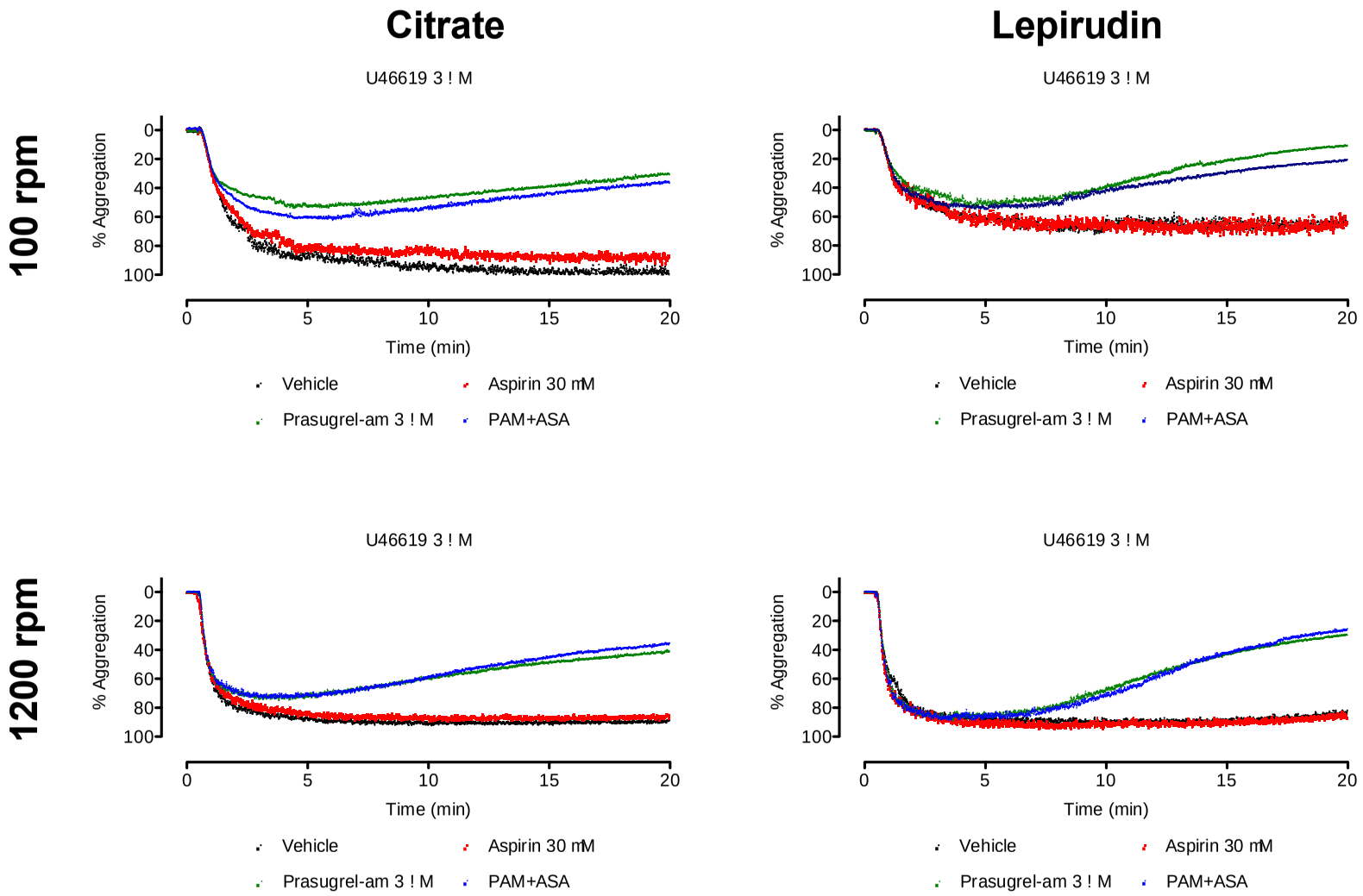

Citrate
Lepirudin
100 rpm
1200 rpm
CRP 100 ng/ml
% Aggregation
0
20
40
60
80
100
0
5
10
15
20
Time (min)
Vehicle
Aspirin 30 mM
Prasugrel-am 3 ! M
PAM+ASA

6.3.5.4 U46619

Final aggregation responses induced by U46619 were quite similar in both citrated and lepirudinised samples. Responses in vehicle treated samples were maximal by 5 minutes and sustained until 20 minutes and these responses were unchanged in the presence of aspirin [figure 6.14]. PAM did have an inhibitory effect on platelet aggregation in response to U46619. Although there was relatively little effect at 5 minutes, by 20 minutes substantial disaggregation had occurred. The effect of PAM was not enhanced by the addition of aspirin [figure 6.14].

Figure 6.14 (opposite page) U46619 induced aggregation in citrate and lepirudin at low and high speeds.

Aggregation traces showing the effects of time, stir-speed (100 or 1200rpm) and anticoagulant (citrate or lepirudin) on platelet aggregation induced by 3µM U46619 determined by traditional LTA. PRP samples were treated with either vehicle (0.1% DMSO), aspirin (30µM), PAM (3µM) or PAM+aspirin. Agonist was added 30 seconds after the start of the assay, which ran for 20 minutes. Data represent the mean response of 4 individuals.


Citrate
Lepirudin
100 rpm
1200 rpm
U46619 3 ! M
% Aggregation
0
20
40
60
80
100
0
5
10
15
20
Time (min)
Vehicle
Aspirin 30 mM
Prasugrel-am 3 ! M
PAM+ASA

6.3.6 Time course of thromboxane production in traditional LTA

Results presented in the previous section indicate that aspirin differentially inhibits aggregation responses in citrate and lepirudin treated PRP, particularly when TRAP-6 amide, collagen or CRP-XL are used as agonists. To further understand the mechanism of this, the time course of TXA_2 production (measured as TXB_2) in response to each agonist was studied by halting cyclo-oxygenase activity at 30 seconds, 1, 2, 10 and 20 minutes in both citrated and lepirudinised samples. As aspirin completely inhibits TXA_2 production, these experiments were conducted in the presence of vehicle and PAM only.

In both TRAP6 amide and collagen treated samples the rate of TXA_2 production was highest in the first two minutes of the aggregation phase, and the peak of thromboxane release was reached by 10 minutes, after which point no further increase in TXA_2 was detected [figures 6.15 and 6.16]. As expected, TXA_2 formation was measurably higher in citrated, compared to lepirudinised, samples and production was suppressed in all samples treated with PAM 3µM [figures 6.15 and 6.16].

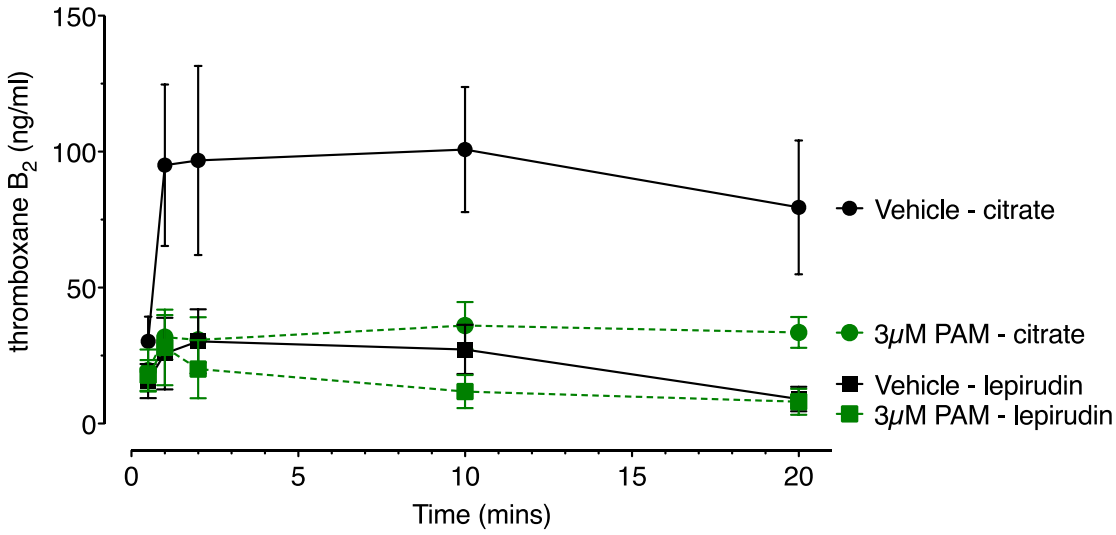


Figure 6.15 Time course of TRAP-6 amide induced TXB_2 production.

TXB_2 production was measured before (30 second time point) and after (1, 2, 10 and 20 minute time points) stimulation of platelets with 3µM TRAP-6 during traditional LTA. At each time point, TXB_2 production was halted by the addition of 1mM diclofenac. Experiments were performed in citrated or lepirudinised PRP treated with either vehicle (0.1% DMSO) or 3µM PAM. Data are mean ± s.e.m. for 4 individuals.

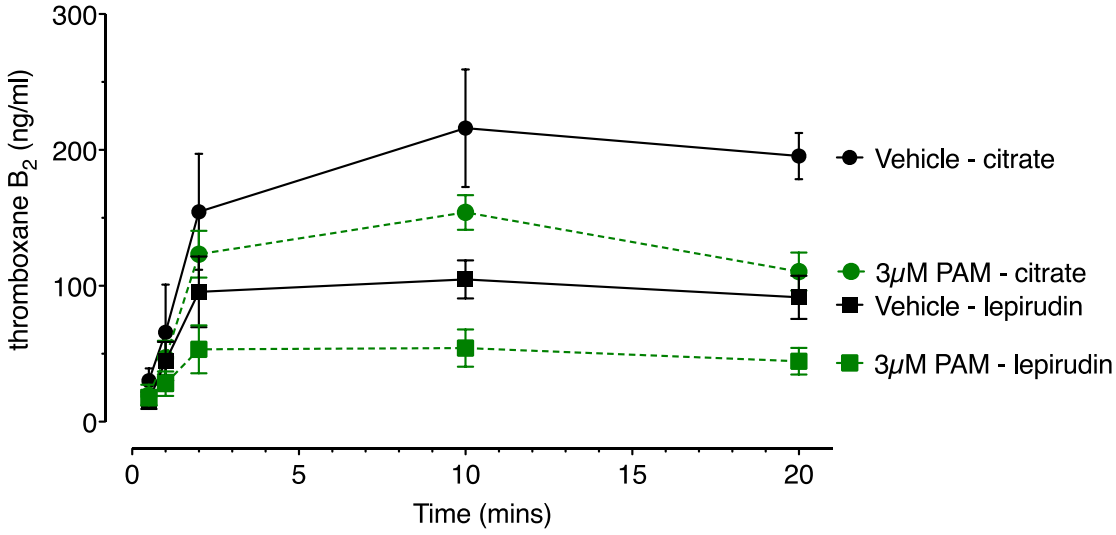


Figure 6.16 Time course of collagen induced TXB_2 production.

TXB_2 production was measured before (30 second time point) and after (1, 2, 10 and 20 minute time points) stimulation of platelets with 3µg/ml collagen during traditional LTA, as for figure 6.15.

Whilst the majority of TXA_2 production after stimulation with CRP-XL occurs in the first two minutes, there is further production up until 20 minutes under control conditions in citrated samples [figure 6.17]. This production is suppressed by PAM. TXA_2 production induced by CRP-XL in lepirudinised samples was very low under both control conditions and in the presence of PAM [figure 6.17].

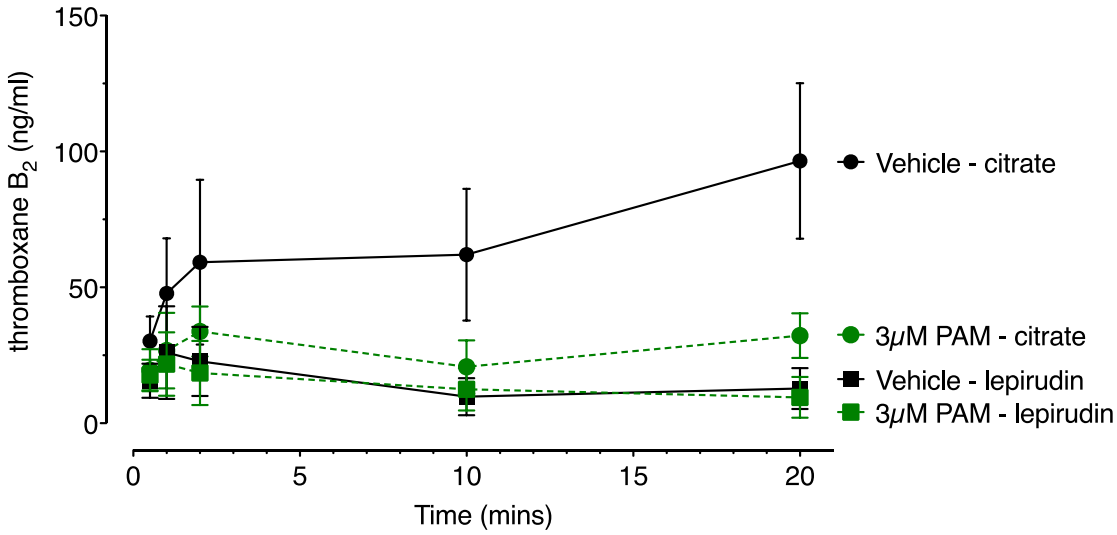


Figure 6.17 Time course of CRP-XL induced TXB_2 production.

TXB_2 production was measured before (30 second time point) and after (1, 2, 10 and 20 minute time points) stimulation of platelets with 100ng/ml CRP-XL during traditional LTA. At each time point, TXB_2 production was halted by the addition of 1mM diclofenac. Experiments were performed in citrated or lepirudinised PRP treated with either vehicle (0.1% DMSO) or 3μM PAM. Data are mean ± s.e.m. for 4 individuals.

6.4 Summary

Systematic investigation of the effects of time, stir-speed and anticoagulant has demonstrated that all of these factors can affect the sensitivity of traditional LTA measurements to anti-platelet drugs.

The initial experiments in this chapter focused on the role of stir-speed, showing that whilst control responses may be similar at both low and high speeds, the sensitivity to aspirin and PAM can vary. This is particularly true for collagen, for which aspirin and PAM alone are effective inhibitors at low speeds although at higher speeds these drugs have limited effects individually. Parallel experiments with CRP-XL suggest that this is not due to any change in GPVI signaling, although TXA_2 production in response to collagen was increased with speed, and TP receptor activation was less inhibited by PAM at higher speeds.

The suggestion that TXA_2 production may be responsible for altering responses to platelet antagonists indicated that experiments should be performed in lepirudin as well as in citrate. Although the extent of collagen-induced aggregation was identical in citrated and lepirudinised samples, the level of TXA_2 production was lower in lepirudin. It is possible that, under control conditions with each anticoagulant, TXA_2 production reaches the threshold required for release of ADP at concentrations that can sustain aggregation. Despite that, PAM had a stronger inhibitory effect in lepirudin, whereas the effect of aspirin was weaker, suggesting a more limited role of TXA_2 under these conditions.

When testing only the GPVI element of collagen-induced platelet activation, by the use of CRP-XL, the production of TXA_2 was found to be much lower although under control conditions a similar level of sustained platelet aggregation was achieved in

both citrated and lepirudinised samples. The pharmacological removal of TXA_2 production by aspirin had a strong effect in citrated samples although a very weak effect in lepirudinised samples, where the production of TXA_2 was already lower. The effect of PAM 3µM in lepirudinised samples, which was to cause near total inhibition of CRP-XL-induced aggregation, suggests that in this system is predominantly ADP dependant. In citrate, where there was residual TXA_2 production, the inhibition of ADP by PAM 3µM had a less complete inhibitory effect on CRP-XL-induced aggregation. It is clear that the additional inhibitory effects of aspirin, when used with PAM, against collagen-induced aggregation are not explained by inhibition of GPVI signaling, except when TXA_2 production is elevated by citrate.

In addition to the stir-speed and choice of anticoagulant, time is an important factor in traditional LTA. Performing traditional LTA for a short period of time shows only the initial response to stimuli, which may or may not be sustained over time. The data presented here demonstrate that in the presence of aspirin and PAM 3µM a number of early responses reverse over time and so early cessation of the aggregation assay may underestimate the effect of these, and other, drugs. This is particularly relevant to the effects of TRAP-6 amide; aggregation to this agent is largely reversible in the presence of PAM, although early cessation of the assay indicates a more limited effect of $P2Y_{12}$ receptor blockade.

The question of which technique, 96-well plate aggregometry or traditional LTA, is more appropriate can be answered quite simply: both are entirely artificial. The selection of optimal aggregometry conditions in traditional LTA was on the basis of producing a maximal response to a fixed concentration of ADP in a short period of time, and this was achieved with higher speeds and higher temperatures [171, 172]. Over time this approach of high speed stirring for a short period of time has been adopted to become normal practice, and this technique does not necessarily reflect

in vivo conditions in any way. Similarly, the conditions used for 96-well plate aggregometry are arbitrary. Both techniques are useful in eliciting normal responses, or the absence of a normal response, which is why aggregometry can be used to detect bleeding disorders associated with hypo-responsiveness to a platelet agonist. However, it has been difficult to ascertain what response should be expected after the administration of anti-platelet drugs, and whether this measurement has any bearing on future clinical events [160]. Problems have arisen from the attempts to use ADP-induced aggregation as a measure of aspirin efficacy [170], and this is unsurprising on reflection of the current literature on this pathway [149]. ADP-$P2Y_{12}$ mediated aggregation is not TXA_2 dependent, unless the platelet sample has been anticoagulated in sodium citrate, and so the contribution of aspirin to inhibit this response is only present under abnormal conditions (low extracellular calcium) [149]. An alternative method of measuring platelet activation may be to assess integrin activation states [173]. The immediate next step with this work will be to study the relative levels of GPIIb/IIIa activation in stirred samples treated with aspirin and strong $P2Y_{12}$ receptor blockade. GPIIb/IIIa activation can be measured by PAC-1 binding [174], which is most easily quantified by flow cytometry [175]. This is possible when platelets have been stimulated in a dilute platelet solution under static conditions to prevent the formation of aggregates [175]. However, this will not serve as a useful model of activation under high speed stirring in aggregometry.

An alternative approach is to measure Rap1b activation. Rap1b is a small GTPase and a critical regulator of GPIIb/IIIa activation [94, 95, 176-178]. It should be possible to conduct LTA under the conditions described in this chapter and by lysing platelets/platelet aggregates at various time points to measure activated Rap1b (GTP-bound). This would allow correlation of GPIIb/IIIa activation with aggregation and TXA_2 production under similar conditions, providing further insight into the effects of aspirin and strong $P2Y_{12}$ receptor blockade in traditional LTA.

Chapter 7: The effect of COX isoform deficiency on prostanoid production

7.1 Introduction

The pro-thrombotic potential of chronic COX inhibition is now well accepted [54, 179], although the mechanisms underlying this effect remain to be fully characterized. One hypothesis is that the increased risk derives from the loss of cardioprotective prostacyclin, which is generated downstream of COX-2 specifically [49], although there is little solid evidence to support this. It has also been suggested that this risk is a composite of the increased risk of hypertension associated with chronic NSAID therapy, and the loss of cardioprotective prostacyclin, which is generated downstream of COX-1 [21].

As explained in chapter 1, COX-1 is believed to be the predominant COX isoform in healthy tissues, and its expression is constitutive, with little dynamic regulation. In contrast, COX-2 is well described as an inducible, early response type protein, which is critical in the development of inflammation in a range of tissues. It is also known that COX-2 is expressed in select tissues, including the kidney and brain, under normal conditions - either constitutively or in response to specific signals.

To investigate more broadly the effect that loss of cyclo-oxygenase function has on prostanoid generation in the cardiothoracic system, and how this could be related to the observed increased risk of thrombosis in populations taking COX inhibitors, I studied the congenital loss of either COX-1 or COX-2 in select organs of genetically modified mice.

7.2 Methods

7.2.1 Animals

C57Bl/6, COX-$1^{-/-}$ and COX-$2^{-/-}$ mice were fed on a standard chow pellet diet and had free access to water. All animals were maintained on a 12 hour light/dark cycle. Animal work was performed according to Home Office regulations in the Animals (Scientific Procedures) Act 1986.

7.2.2 Acute systemic inflammation model

To model acute systemic inflammation WT, COX-1 -/- and COX-2 -/- mice were administered 10mg/kg LPS by intraperitoneal injection and regularly monitored for signs of severe distress. Four hours later, mice were killed by rising concentrations of CO_2 and blood and organs collected as required.

7.2.3 Whole blood stimulation assay

LPS treated mice as described above [section 7.2.2] and control, untreated mice, were used in this assay. After killing, blood was immediately drawn from the inferior vena cava into a syringe containing sodium heparin to give a final concentration of 10U/ml. Heparinised whole blood was transferred to a microcentrifuge tube and incubated for 30 minutes at 37ºC in the presence of 50µM A23187, a calcium ionophore. After 30 minutes, diclofenac was added to a final concentration of 1mM and the whole blood was spun at 1300 g to separate cellular material from the plasma supernatant. The supernatant was withdrawn and stored at -80ºC. Supernatant TXB_2 concentrations were determined by the method described in section 2.8.2.

7.2.4 LPS stimulation of J774.2 cells and preparation of cell lysate

J774.2 murine macrophages (European Collection of Cell Cultures) were seeded into six-well tissue culture plates and incubated with either medium containing 1 µg/ml LPS or medium alone. After 24-48 hours medium was removed and cells washed twice with ice-cold PBS before the addition of ice-cold lysis buffer containing EDTA (10 mM), 1% v/v Triton-X 100, PMSF (1 mM) and Protease inhibitors (Protease inhibitor cocktail, Roche). Lysed cells from each well were recovered and pooled before protein determination by Bradford assay. Cell lysates were stored at -80°C until required and then further prepared by dilution in PBS to a protein concentration of between 1.5 and 3 mg/ml before boiling 1:1 with Laemmli buffer.

7.2.5 Preparation of organ homogenates for Western blotting

Organs were weighed, placed in 1:5 (weight:volume) ice-cold lysis buffer (as described in 7.2.2) and homogenised. Protein concentration was determined by Bradford assay and homogenates stored frozen at -80°C until use, and then prepared as for cell lysates (see above).

7.2.6 Western blotting

Samples containing equal protein, typically 40-60 µg, were loaded onto 7.5% polyacrylamide gels alongside Rainbow molecular weight markers (Amersham Biosciences) for separation by electrophoresis in a Tris/Glycine/SDS buffer. Proteins were transferred to a PVDF membrane that was cut in two at the level of the 52 kDa marker and blocked in a 5% Milk/0.1% PBS-Tween buffer for 1 hour at room temperature. Membranes were probed with specific rabbit polyclonal antibodies against COX1 (CAY160109; Cayman Chemicals, Ann Arbor, MI, USA), COX2 (CAY160126; Cayman Chemicals) and specific mouse monoclonal antibody for beta-actin (Abcam, UK) overnight at 4°C. After washing, membranes were further

incubated with goat polyclonal anti-rabbit or anti-mouse HRP linked antibodies, as appropriate. ECL reagent was added to each membrane before visualisation of the blot image by exposure to film.

7.2.7 Western blotting image analysis

Western blot images were scanned (Canon, CanoScan LiDE80) and digital images analysed with Bio-Rad Quantity One v4.6.6 (Bio-Rad Laboratories Inc.,) to quantify the density of relevant immunoreactive protein bands.

7.2.8 Prostanoid generation in whole organ homogenates

Time out of animal before homogenization: 45 minutes (maximum)

Organs from Wild type, COX-1-/- and COX-2-/- mice were dissected and immediately placed in 1:5 (weight:volume) ice-cold Tris buffer (50 mM, pH 7.4) containing PMSF (1 mM). Organs were then lysed using an automated ceramic bead homogeniser (Bertin Precellys 24) and incubated for 20 minutes at 37°C. Diclofenac (1 mM) was added and homogenates incubated for a further 10 minutes, at 37°C. Homogenates were centrifuged and the supernatant stored at -80°C for later radioimmunoassay.

7.2.9 Prostanoid generation in sectioned tissues

Time out of animal: 2 hours 30 minutes to complete experiment

WT, COX1 -/- and COX2 -/- mice were euthanized with carbon dioxide and ventricles/right lung (along with aorta, kidneys etc.) removed immediately into PBS. Sections of lung and left ventricle wall were then rested at 37˚C in individual wells of a 96-well plate containing 200µl DMEM in the presence or absence of diclofenac (100µM), each in duplicate. After 60 minutes, the content of each well was aspirated and sections further incubated with fresh media (200µl/well). 30 minutes later a pre-

stimulation sample was taken (50µl) from each well and stored at -80˚C. The volume in each well was restored to 200µl by the addition of media with or without diclofenac (100µM) also containing either A23187 (50µM in 0.25% DMSO final) or vehicle (0.25% DMSO final). After 30 minutes incubation (37˚C) a post-stimulation sample was taken and stored at -80˚C for later enzyme immunoassay.

7.2.10 Enzyme immunoassay for 6-keto $PGF_{1\alpha}$

Samples were assayed either 1:2 (all COX1-/- samples, WT and COX2-/- samples with diclofenac) or 1:20 (WT and COX2-/- samples without diclofenac) in assay buffer and the assay performed as described in the kit instructions (6-keto prostaglandin $F_{1\alpha}$ enzyme immunoassay kit, Catalogue no. 515211, Cayman Chemicals, Ann Arbor, MI, USA). Briefly, samples and known standards were assayed alongside a maximum binding control on a 96-well microtitre plate, coated with goat anti-rabbit IgG antibodies. The plate was incubated at 4ºC for 18 hours after the addition of a 6-keto-$PGF_{1\alpha}$-acetylcholinesterase conjugate and 6-keto-$PGF_{1\alpha}$-specific rabbit antiserum. After washing, Elmann's reagent solution was added, the plate was covered and allowed to develop for 90-120 minutes on an orbital shaking platform. Once developed, the absorbance of each well was determined at 405nm. From the absorbance data, the percentage binding of known standards was calculated in reference to the maximum binding (zero 6-keto-$PGF_{1\alpha}$) control wells, plotted against the logarithm of concentration and analysed by non-linear regression using a four-parameter logistical fit model. Unknown samples were expressed in a similar fashion, interpolated from this standard curve and corrected for dilution.

7.2.11 Inflammatory cytokine assay

LPS treated mice as described in section 7.2.2 and control, untreated mice were used in these experiments. After killing, blood was immediately drawn from the

inferior vena cava into a syringe containing sodium heparin to give a final concentration of 10U/ml. Whole blood was transferred to a microcentrifuge tube and spun at 1300 x g to separate cellular material from the plasma supernatant. The supernatant was withdrawn and stored at -80ºC until the cytokine assay was performed.

Plasma inflammatory cytokines were determined using the MSD MULTI-SPOT® 7-plex pro-inflammatory cytokines 96-well plate assay (K11012C-1, Meso Scale Discovery, Gaithersburg, MD, USA) in accordance with the manufacturers guidelines. Briefly, diluted plasma samples (10µl/well) were incubated at room temperature on a 7-plex MULTI-SPOT plate alongside a calibrator mix (known standards) for 2 hours, with vigorous shaking. Each well was washed three times before the addition of a detection antibody solution. After a further 2 hour incubation at room temperature, a specific 'read buffer' was added and the plate analysed on a MSD SECTOR 6000 instrument (Meso Scale Discovery, Gaithersburg, MD, USA). Unknown samples were interpolated from the standard curve and corrected for dilution.

7.2.12 PGE_2 HTRF assay

This assay works on the same principle as the TXB_2 HTRF assay described in section 2.8.3, using d2-labeled PGE_2 and an anti-PGE_2 antibody labeled with cryptate.

Before the assay was run samples were diluted in assay diluent (50mM phosphate buffer, pH7.0 containing 0.2% BSA). PGE_2 standards were similarly prepared by dilution in assay diluent. 10µl diluted supernatant or standard was then added to wells of a 384-well black microtitre plate. 5µl PGE_2-d2 conjugate and 5µl anti-PGE_2

cryptate conjugate were added to each well and the plate incubated at room temperature for 1hr. A positive (10µl diluent, 5µl d2 conjugate, 5 µl cryptate conjugate) and negative control (10µl diluent, 5µl buffer, 5µl cryptate) were included on each assay plate. After excitation at 320nm, emission at 665nm and 620nm was determined for each well. The mean 665/620nm ratio of each sample/control was then calculated from individual replicates and used to calculate the Delta $F_{\%}$ for each sample/standard, where Delta $F_{\%}$ = $((Ratio_{sample}-Ratio_{neg\ control})/Ratio_{neg\ control})*100$.

Delta $F_{\%}$ values were plotted against the logarithm of concentration for known standards and analysed by non-linear regression using a four-parameter logistical fit model. Unknown samples were interpolated from this standard curve and corrected for dilution.

7.2.13 Statistical analysis

Data presented in column charts were analysed in GraphPad Prism [section 2.10] by one-way ANOVA with Bonferroni's post-test. Statistical significance was considered to be a p-value≤0.05.

7.3 Results

7.3.1 The production of thromboxane in whole mouse blood is COX-1 dependent

Stimulation of whole mouse blood from wild type, COX-1 and COX-2 deficient mice indicated that TXA_2 production is wholly dependent on COX-1 in both healthy animals and those pre-treated with 10mg/kg LPS to model systemic inflammation [figure 7.1]. In those mice treated with LPS the level of TXA_2 production was partially reduced.

7.3.2 COX isoform expression in tissues relevant to the cardiothoracic system

The relative levels of COX isoform expression were determined, by western blotting, in the heart, lung, kidney and brain of WT, COX-1 and COX-2 deficient mice. The confirmation of immunoreactive COX was obtained by comparing the signal from mouse tissues to that of rat kidney, which expresses both COX-1 and COX-2 immunoreactive proteins. To confirm COX-2 expression, rat kidney homogenates were blotted alongside naïve and LPS-treated J774.2 cells. COX-2 protein has a molecular weight of 70 kDa and so is expected to migrate with protein markers close to this weight. As figure 7.2 shows, COX-2 immunoreactive protein from rat kidney and LPS stimulated J774.2 cells co-migrated between the 52 and 76 kDa markers. Similarly, COX-1 immunoreactive protein was detected in the same molecular weight region in both rat kidney and J774.2 cells.

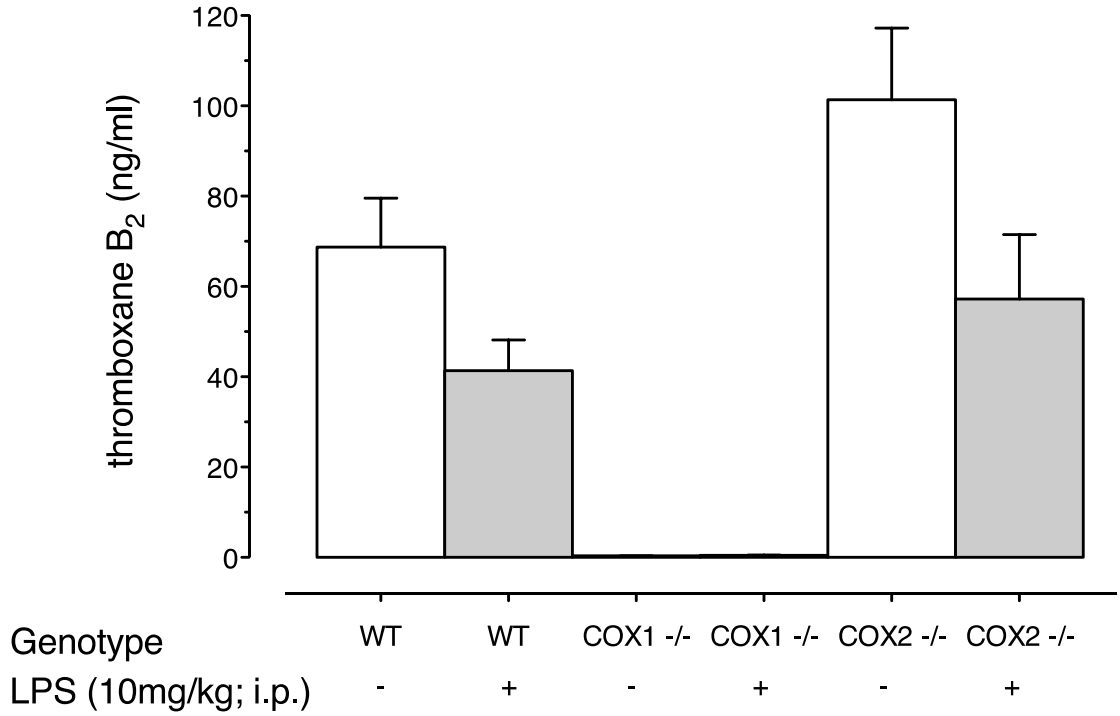


Figure 7.1 TXB_2 production stimulated by A23187 in whole blood from wild type and COX-1/-2 deficient mice.

TXB_2 production in whole blood from wild type (WT), COX-1 deficient (COX1-/-) and COX-2 deficient (COX2-/-) mice with and without intraperitoneal administration of LPS (10mg/kg) four hours before blood collection. TXB_2 production was stimulated by the addition of 50µM A23187, a calcium ionophore. After 30 minutes incubation at 37°C, COX activity was halted by the addition of diclofenac to a final concentration of 1mM. Data are mean ± s.e.m for 4 animals per group.

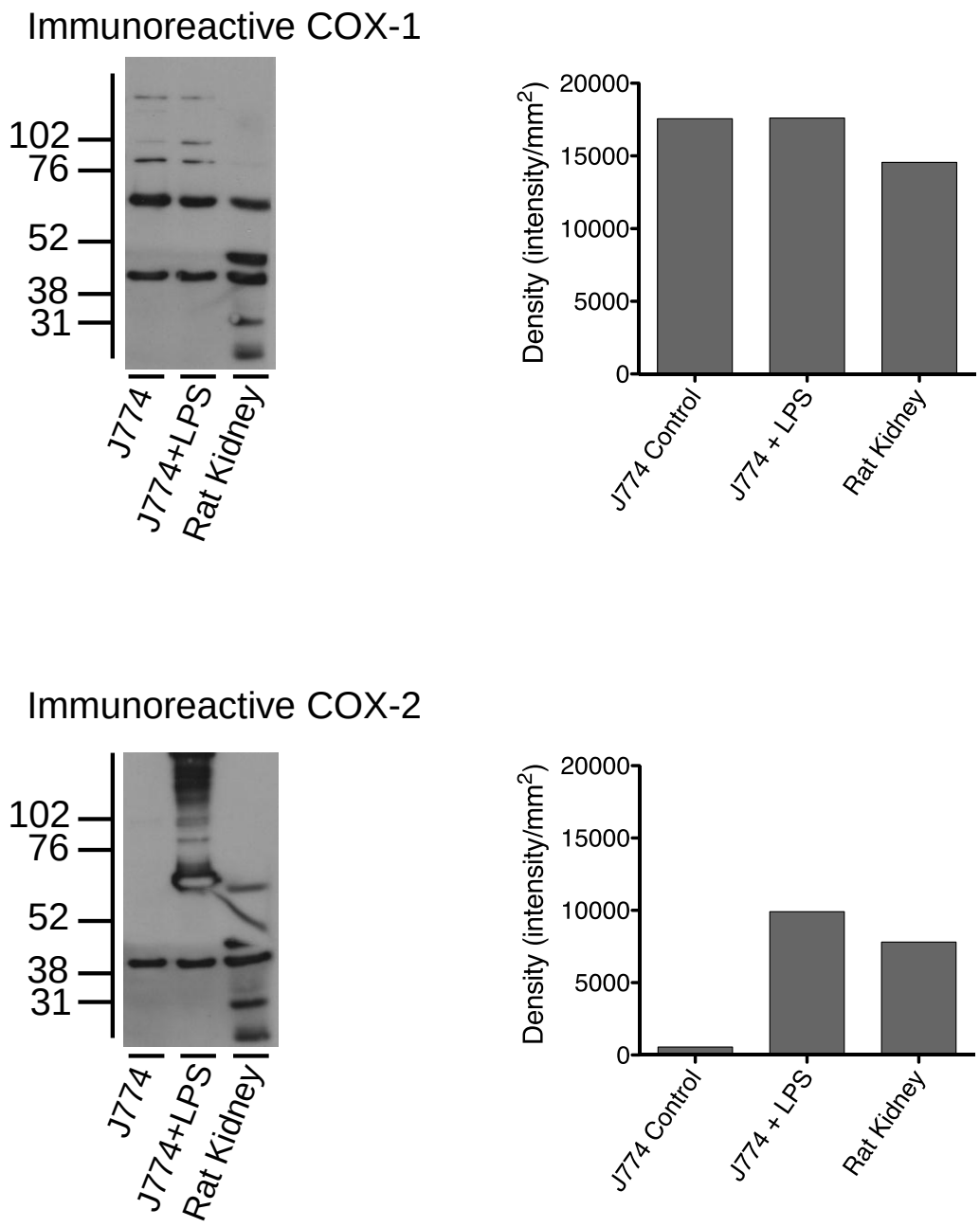


Figure 7.2 COX isoform immunoreactivity in J774.2 cells and rat kidney.

Left hand column, detection of either COX-1 or COX-2 by western blotting of homogenized J774.2 cells, with or without incubation with 1µg/ml LPS, and rat kidney. Both COX-1 and COX-2 are identifiable as bands appearing between the 52 and 76kDa molecular weight markers. Right hand column, relative density of bands identified as representing either COX-1 or COX-2.

COX-2 protein was not detected in any mouse tissue, although immunoreactive protein was consistently detected in the rat kidney control samples [figure 7.3].

COX-1 was detected constitutively in all organs of both WT and COX-2 deficient mice [figure 7.4]. Whilst the anti-COX-1 primary antibody used in this study detected no bands in homogenates of the kidney or brain of COX-1 deficient mice, in heart and lung homogenates a residual band was detected just below the COX-1 protein. Based on the functional data outlined below, this residual band is not thought to represent COX-1 protein.

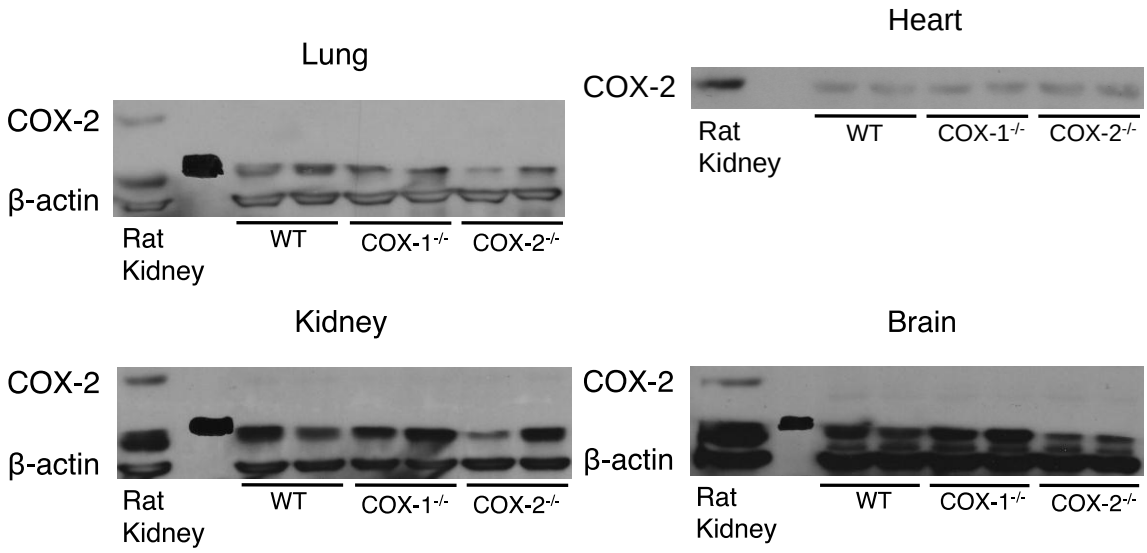


Figure 7.3 COX-2 immunoreactive protein in tissues from wild type and COX-1/-2 deficient mice.

Typical blot images representing detection of COX-2 and β-actin by western blotting of homogenized lung, heart, kidney and brain from wild-type (WT), COX-1 deficient (COX-1-/-) and COX-2 deficient (COX-2-/-) mice aged 12-16 weeks. Homogenised rat kidney is included as a positive control. β-actin was not detectable in the mouse heart.

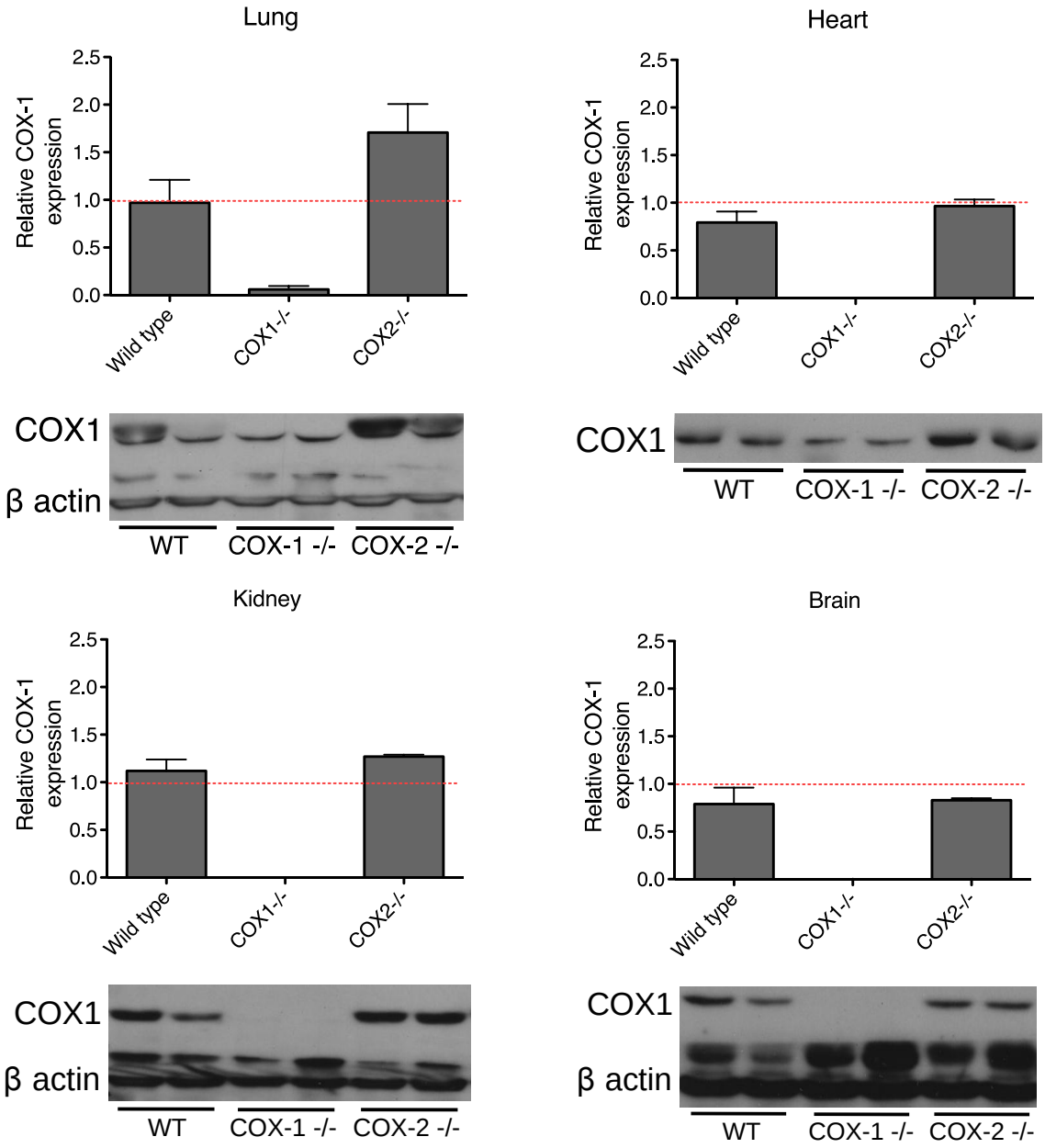


Figure 7.4 COX-1 immunoreactive protein in tissues from wild type and COX-1/-2 deficient mice.

Detection of COX-1 and β-actin by western blotting of homogenized lung, heart, kidney and brain from wild-type (WT), COX-1 deficient (COX-1-/-) and COX-2 deficient (COX-2-/-) mice aged 12-16 weeks. Relative COX-1 expression is the density of the COX-1 band normalised to the density of the equivalent β-actin band. β-actin was not detectable in the mouse heart and so heart data are normalized to the average density of lung β-actin bands. Column chart data are mean ± s.e.m. for 2-4 animals per group.

7.3.3 The relationship between COX isoforms and prostacyclin production in healthy mouse tissues

The effect of losing functional COX-1 or COX-2 protein on the production of prostacyclin was assessed using crude whole organ homogenate and sectioned tissues models. The crude whole organ homogenate model showed that in the heart, lung, kidney and brain prostacyclin production was overwhelmingly COX-1 dependent [figure 7.5]. In fact, whilst prostacyclin generation was all but abolished by the loss of COX-1, the loss of COX-2 did not reduce prostacyclin production below the levels seen in WT animals ($p>0.05$). Intriguingly, the loss of COX-2 in the lung resulted in an increase in prostacyclin production above that seen in WT mice ($p<0.05$) [figure 7.5].

As crude whole organ homogenates only give an overview of the maximum possible production in disrupted tissue this model may exaggerate the effects of COX-1 if the tissue protein levels are normally substantially higher than those of COX-2. Therefore, the prostacyclin production in sectioned heart and lung tissue was also studied. When assessed by this method, prostacyclin production was substantially lower, although once again it was entirely COX-1 dependent [figure 7.5]. Even when sections of left ventricle and lung were stimulated with the calcium ionophore, A23187, to promote the generation of arachidonic acid, there was no change in prostanoid production in any sample ($p>0.05$) [figure 7.6].

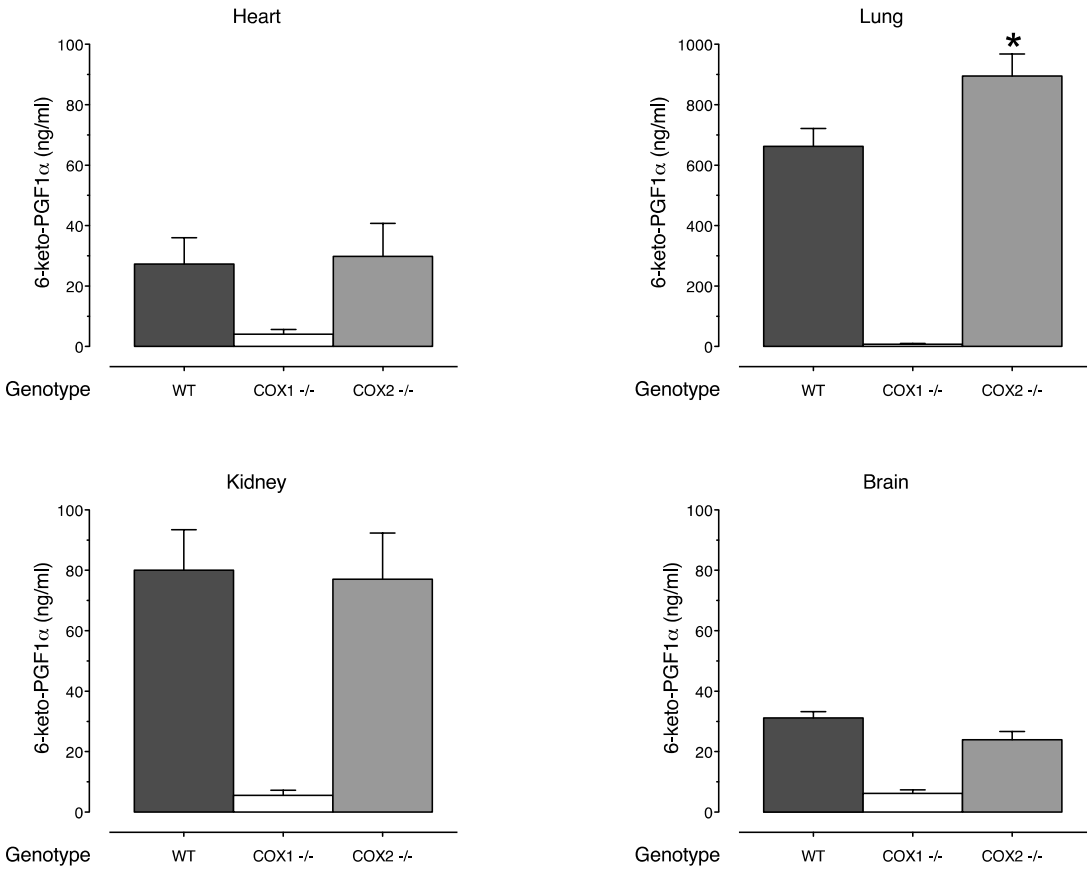


Figure 7.5 PGI_2 production in crude whole organ homogenates of wild type and COX-1/-2 deficient mice.

Lungs, hearts, kidneys and brains from wild-type (WT), COX-1 deficient (COX-1-/-) and COX-2 deficient (COX-2-/-) mice were homogenised in 50mM Tris buffer, containing 1mM PMSF, then incubated at 37°C for 20 minutes to allow 6-keto $PGF_{1\alpha}$, a stable metabolite of PGI_2, to accumulate. After 20 minutes, COX activity was halted by the addition of diclofenac to a final concentration of 1mM. After a further 10 minutes, homogenates were centrifuged and the supernatants removed for evaluation of 6-keto $PGF_{1\alpha}$ formation by enzyme-linked immunoassay. Data represent mean ± s.e.m. for 6 animals per group. * indicates $p<0.05$ compared to wild-type by one-way ANOVA.

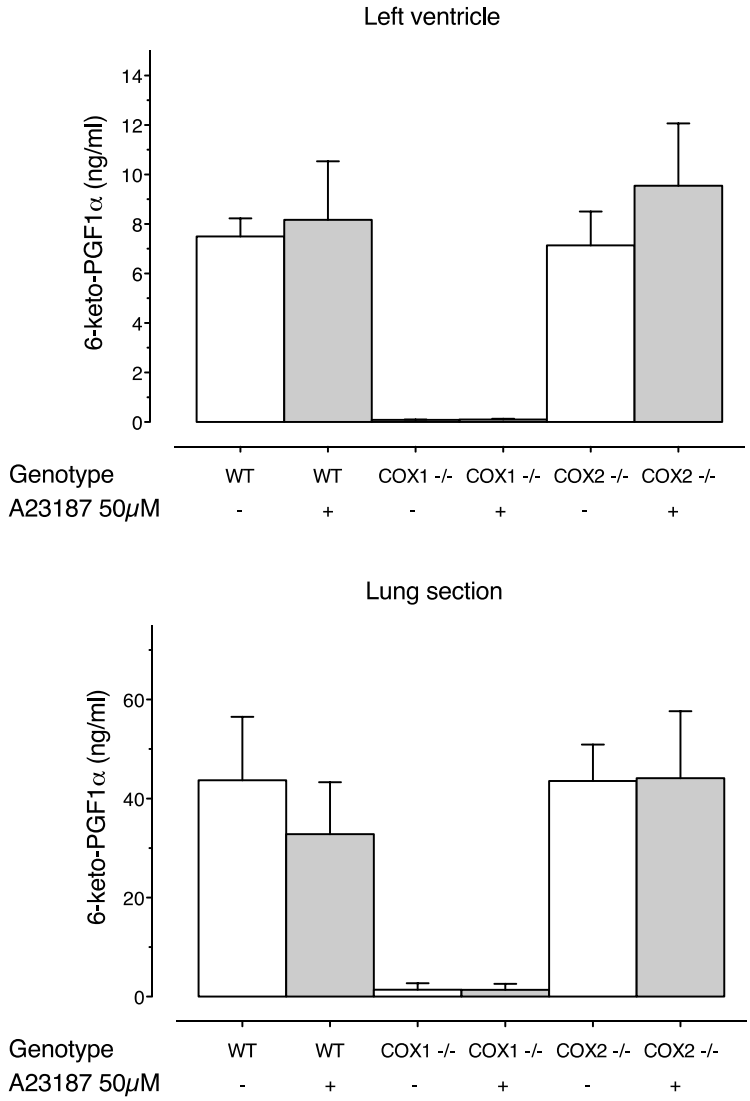


Figure 7.6 PGI_2 production in dissected left ventricle and lung from wild type and COX-1/-2 deficient mice.

Hearts and lungs from wild-type (WT), COX-1 deficient and COX-2 deficient mice were sectioned and then rested at 37˚C in individual wells of a 96-well plate containing 200µl DMEM. After 60 minutes, the content of each well was aspirated and sections further incubated with fresh media (200µl/well). 30 minutes later 50µl media was removed from each well and the volume restored to 200µl by the addition of media containing either 50µM A23187 (in 0.25% DMSO) or 0.25% DMSO. After 30 minutes incubation (37˚C) a sample was taken and stored at -80˚C for later determination of 6-keto $PGF_{1\alpha}$ by enzyme-linked immunoassay. Data represent mean ± s.e.m. for 6 animals per group. * indicates $p<0.05$ compared to equivalent wild-type by one-way ANOVA.

7.3.4 The effect of COX isoforms on inflammation in the cardiothoracic system

As shown in figure 7.1, the COX-dependency of TXA_2 production in whole blood is unaltered by systemic inflammation. It is unclear as to why the magnitude of TXA_2 production is decreased with LPS treatment and possible reasons for this are discussed in the summary [section 7.4].

Measurement of plasma cytokines confirmed that intra-peritoneal administration of 10mg/kg LPS resulted in systemic inflammation in WT, COX-1-/- and COX-2-/- mice. Of the seven pro-inflammatory cytokines measured, all were detected at equivalent low concentrations in untreated mice, suggesting no effect of COX isoforms on their expression under basal conditions. For the majority of cytokines (IL-1beta, TNFα, KC, IL-6 and IL-12p70), there were no differences in cytokine levels between COX mutant mice and WT mice [figure 7.7]. However, IFNγ and IL-10 plasma levels appear to be partially regulated by COX isoforms. IFNγ was substantially decreased ($p<0.01$) in COX-1, but not COX-2 mutant mice, compared to WT, whereas IL-10 levels were slightly depressed in COX-2,-/- although not COX-1-/- ($p<0.05$) [figure 7.7].

In this model of systemic inflammation, production of prostacyclin and PGE_2 was measured from crude whole organ homogenates. Prostacyclin production in the heart, lung, kidney and brain was unaffected by a strong inflammatory stimulus and remained to be COX-1 driven [figure 7.8]. PGE_2 production was similar, and although there was a tendency for the production of this prostanoid to be elevated in the heart, kidney and brain of inflamed WT and COX-2 deficient mice these differences did not reach significance ($p>0.05$) [figure 7.9].

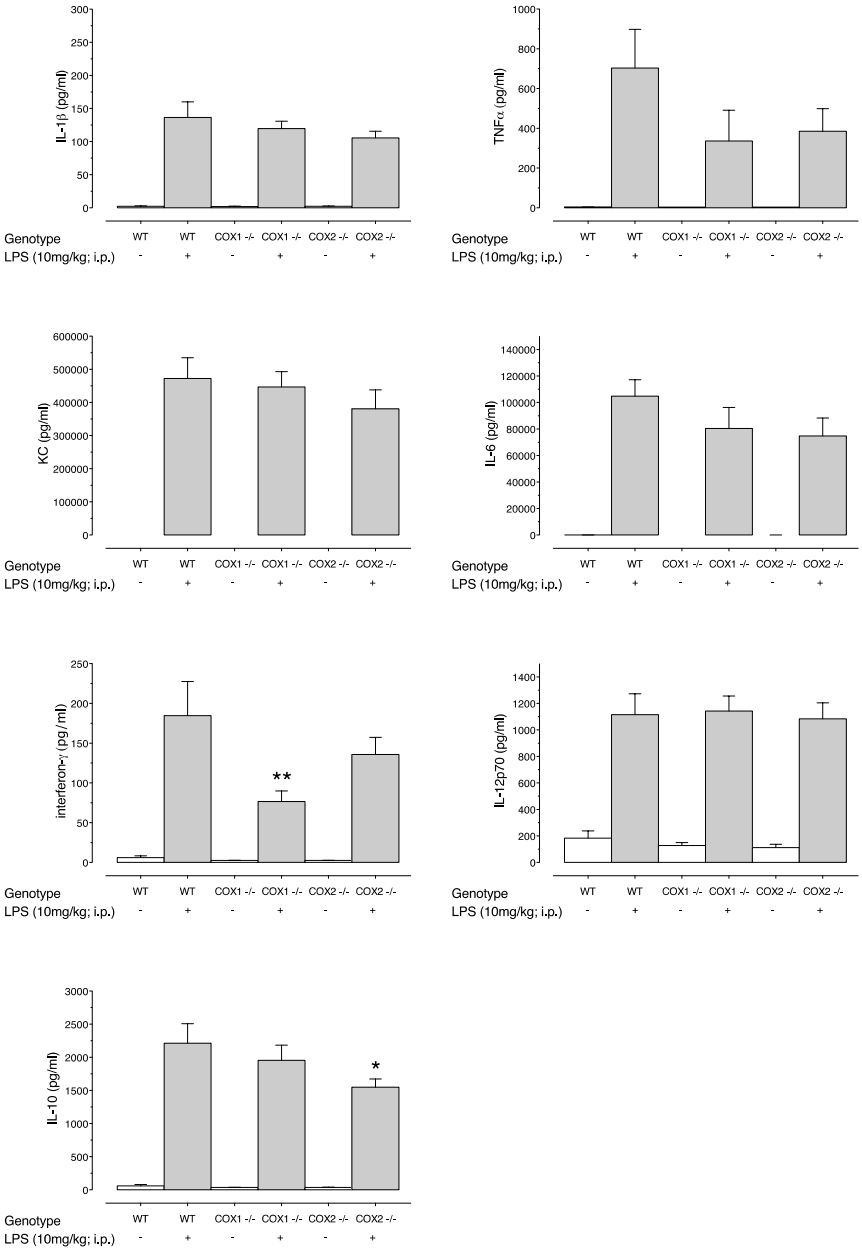


Figure 7.7 Plasma cytokine production in healthy and inflamed wild type and COX-1/-2 deficient mice.

Plasma levels of IL-1β, TNFα, KC, IL-6, IFNγ, IL-12p70 and IL-10 were determined by mesoscale discovery multispot assay in wild-type (WT), COX-1 deficient (COX-1-/-) and COX-2 deficient (COX-2-/-) mice four hours after intraperitoneal administration of LPS (10 mg/kg). Control values from untreated mice are also shown. Data represent mean ± s.e.m. for 6-12 animals per group. * indicates $p<0.05$, ** $p<0.01$, compared to equivalent wild-type by one-way ANOVA.

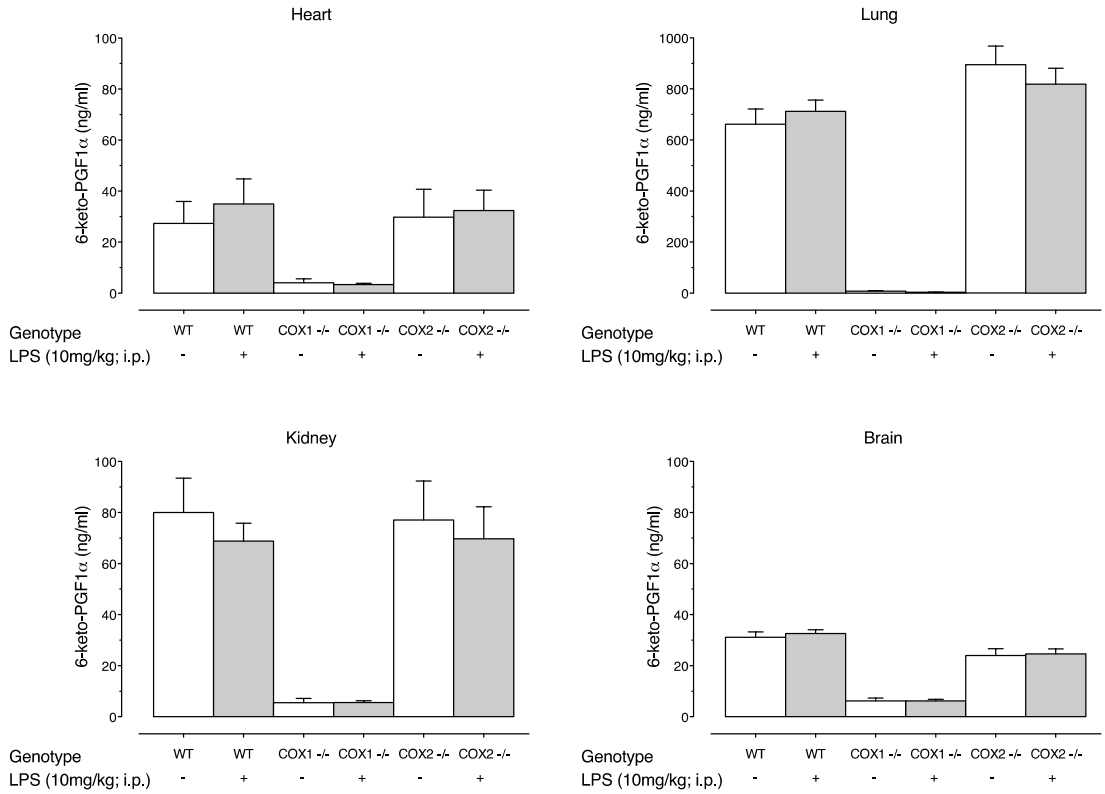


Figure 7.8 PGI_2 production in crude whole organ homogenates from healthy and inflamed wild type and COX-1/-2 deficient mice.

Lungs, hearts, kidneys and brains were collected from wild-type (WT), COX-1 deficient (COX-1-/-) and COX-2 deficient (COX-2-/-) mice four hours after intraperitoneal administration of LPS (10mg/kg) or from equivalent control animals. Organs were homogenised in 50mM Tris buffer, containing 1mM PMSF, then incubated at 37°C for 20 minutes to allow 6-keto $PGF_{1\alpha}$, a stable metabolite of PGI_2, to accumulate. After 20 minutes, COX activity was halted by the addition of diclofenac to a final concentration of 1mM. After a further 10 minutes, homogenates were centrifuged and the supernatants removed for evaluation of 6-keto $PGF_{1\alpha}$ formation by enzyme-linked immunoassay. Data represent mean ± s.e.m. for 6 animals per group (3 male, 3 female). * indicates $p<0.05$ compared to equivalent wild-type by one-way ANOVA.

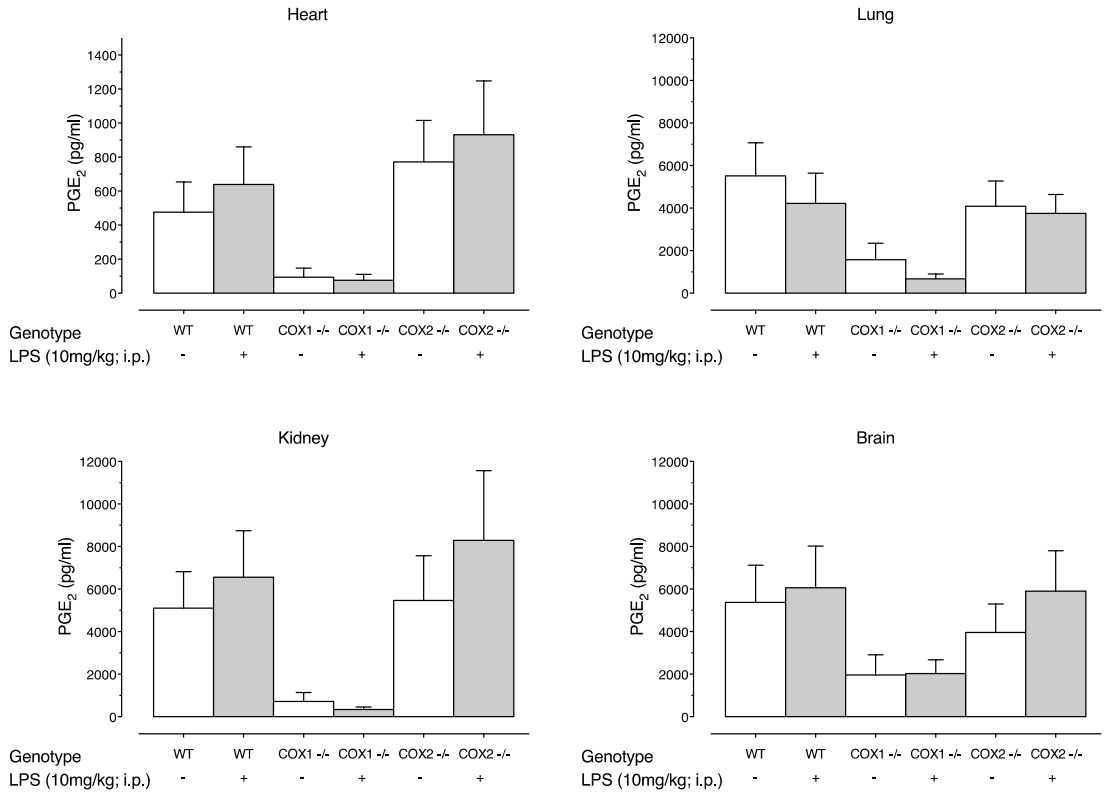


Figure 7.9 PGE2 production in crude whole organ homogenates from healthy and inflamed wild type and COX-1/-2 deficient mice.

Lungs, hearts, kidneys and brains were collected from wild-type (WT), COX-1 deficient (COX-1-/-) and COX-2 deficient (COX-2-/-) mice four hours after intraperitoneal administration of LPS (10mg/kg) or from equivalent control animals. Organs were homogenised in 50mM Tris buffer, containing 1mM PMSF, then incubated at 37°C for 20 minutes to allow PGE_2 to accumulate. After 20 minutes, COX activity was halted by the addition of diclofenac to a final concentration of 1mM. After a further 10 minutes, homogenates were centrifuged and the supernatants removed for evaluation of PGE_2 formation by HTRF assay. Data represent mean ± s.e.m. for 6 animals per group (3 male, 3 female). * indicates $p<0.05$ compared to equivalent wild-type by one-way ANOVA.

7.4 Summary

The functional and expression studies presented in this chapter suggest that COX-1 is the dominant isoform in health and during acute inflammation. The assays used, and the scope of these experimental finding are discussed below.

Ex vivo COX activity in heparinised whole blood, measured as TXA_2 production, was consistently reduced after *in vivo* LPS treatment of the donor mice. This assay used a calcium ionophore to activate blood cells by promoting an inward flux of extracellular calcium ions, which are needed for the activity of cytosolic phospholipases [180]. Reduction of TXA_2 formation by an inhibitory mechanism would therefore suggest a modification of the phospholipase system. This seems unlikely as LPS upregulates cytosolic PLA_2 activation and pro-inflammatory lipids via TLR4 signaling pathways [181], and there are no described mechanisms for the downregulation of phospholipases after LPS treatment. An alternative explanation for the observed reduction in TXA_2 production may be that fewer TXA_2 producing cells are present in the blood of LPS treated mice, particularly macrophages and platelets. As the LPS dose was administered intraperitoneally it is possible that by four hours many previously circulating macrophages have migrated to the peritoneal space, or other sites of inflammation. Additionally, platelets may have become activated in the circulation and sequestered in small aggregates that have been removed from the circulation. This could be further investigated by hematological cell counts in freshly drawn samples to ascertain the proportions of platelets and leukocytes in the presence and absence of LPS-induced systemic inflammation.

Although immunoreactive COX-2 was not detected in either the lung, heart, kidney or brain of the wild type and COX deficient mice this does not entirely rule out the presence of this isoform in these organs. It has been inferred from pharmacological

studies that COX-2 is important for the generation of vasoprotective prostanoids in the vascular endothelium [50, 51]. The lung is highly vascularised and, if this inference is correct, could be expected to contain a substantial amount of COX-2. However, COX-2 was not detected in the lungs of the mice we studied and so is perhaps not present in large amounts. Whilst COX-2 is expressed in the kidneys of both rats and humans previous studies have failed to detect immunoreactive COX-2 protein in the kidneys of C57/Bl6 mice, although COX-2 mRNA is found [182]. It is unclear whether the relative levels of COX-2 are too low to detect, as expression in other species is known to be limited to discrete regions [183-185], or if this isoform is just not translated from the message in this tissue.

It is possible that COX-2 was expressed in the heart and brain of the mice we studied, although only in small discrete regions making it undetectable in a gross organ homogenate. In fact, the presence of COX-2 in the human and rat brains is well documented and is localised to specific regions [186]. I found that COX-1 deficiency in the brain did not completely inhibit the formation of PGE_2, which agrees with work by Bosetti and colleagues showing that COX-2 regulates PGE_2 production in the mouse brain [187]. The presence of COX-2 in the mouse heart has also been reported by others [188].

If COX-2 was present in the tissues studied, the data from the functional assays used here do not support a major role for this isoform in the production of prostanoids on a gross level in the various organs tested. The COX activity of whole organ homogenates was in no way impaired in COX-2 deficient mice, in stark contrast to the effects of COX-1 deficiency. Of course, the functional assay used here only reflects the maximal prostanoid production capacity of these tissues after disruption of structures and liberation of enzymes to allow potentially abnormal substrate-enzyme interactions. For instance, these results do not reflect the regulation that may be imposed upon endoperoxidases downstream of COX and the governance of

association between these enzymes and the COX isoforms [189, 190]. However, even in sectioned heart and lung tissue there was a predominance of COX-1 activity.

If both PGI_2 and PGE_2 are in fact regulated by COX-1 in these tissues this could be important in understanding the regulation of vascular and platelet function. Whilst the importance of PGI_2 has been discussed in earlier chapters of this thesis the role of PGE_2 on platelet function has not been covered, and is less well defined. PGE_2 activity can be determined by the distribution of EP receptor subtypes, and where multiple receptor types are expressed receptor selectivity may be determined by the ligand (PGE_2) concentration [21]. Therefore, the effects of PGE_2 on the platelet may be either pro-aggregatory via the EP_3 receptor or anti-aggregatory, acting through the EP_2 or EP_4 receptors [191, 192]. PGE_2 is likely to be important to responses during inflammation as it is known to be upregulated in chronic vascular inflammation [193]. In my experiments there was a trend for an increase in PGE_2 production by the heart and kidney after treatment with LPS, although this did not reach significance. Attempts were made to measure plasma PGE_2 although this was not possible due to the weakness of the signal. This was unsurprising as PGE_2 is known to have a short half-life [194] and its in vivo production may best be measured by a specific urinary metabolite [195], as I reported in Chapter 6 for PGI_2 and TXA_2.

Upregulations of the pro-inflammatory cytokines IL-1β, IL-6, KC and TNFα by LPS treatment were unaltered in either COX-1 or COX-2 deficient mice. Anecdotally, COX-2 deficient mice appeared to display less piloerection and moved more freely after LPS administration, suggesting an absence of fever in these animals. This is consistent with the central regulation of LPS-induced fever by PGE_2, which is reported to be associated with COX-2 [196].

The induction of some anti-inflammatory cytokines was limited by the loss of either COX-1 or COX-2. Interferon gamma production was significantly lower in COX-1

deficient than in either wild type or COX-1 deficient mice, whereas IL-10 was slightly (although significantly) reduced in COX-2 deficient mice. The mechanisms and relevance of these changes are unclear.

Overall these data suggest that COX-1 deficiency may promote the development of inflammation by the loss of anti-inflammatory cytokines, which may contribute to thrombotic vascular events when coupled with a loss of prostanoid activity.

Chapter 8: General Discussion

This thesis has aimed to investigate how inhibition of cyclo-oxygenases in the cardiothoracic system can be pro-thrombotic, with a focus upon how this may help us understand how aspirin acts in combination with $P2Y_{12}$ receptor blockers. To further understand these data, the role of COX-1 and COX-2 isoforms in normal and inflamed conditions has been investigated in genetically modified mouse models.

In chapters 3 and 4 the use of 96-well plate aggregometry has demonstrated that this technique is suitable to test the *in vitro* anti-platelet effects of aspirin as well as PAM and ticagrelor. Using this approach it has been possible to demonstrate that in the presence of strong platelet $P2Y_{12}$ receptor inhibition aspirin has little further anti-aggregatory effect. In addition, and perhaps of greater importance in understanding how these *in vitro* data relate to the clinical use of $P2Y_{12}$ receptor blockers (particularly clopidogrel), the data presented in chapter 4 indicate that aspirin has a beneficial anti-aggregatory effect in the presence of partial $P2Y_{12}$ receptor blockade.

Of critical importance to the interpretation of data from this model is the relevance of the drug concentrations used in my *in vitro* tests to the plasma concentration of active drugs that follow *in vivo* dosing. As has been stated in the relevant chapter summaries, there is good reason to believe that the concentrations used here reflect the concentrations achieved in man taking these drugs as an anti-platelet therapy and data presented in chapter 5 confirm that a strong effect in this assay is achievable with standard dosing in healthy male subjects. As both aspirin and prasugrel are irreversible inhibitors of COX and the $P2Y_{12}$ receptor, respectively, reversal of their effects requires turnover of new platelets. Under normal conditions, this is estimated to occur at a rate of approximately 10% of circulating platelets per day, indicating the need for daily dosing. Ticagrelor is a reversible $P2Y_{12}$ inhibitor, the effects of which will be lost over time [137]. Fortunately, ticagrelor has a long half-life in both healthy volunteers and patients with atherosclerotic disease [137], with little

loss of efficacy 12-16 hours after the last dose following the standard anti-thrombotic dosing regimen [138].

When this *in vitro* evidence, suggesting that strong $P2Y_{12}$ receptor blockade alone has a considerable anti-platelet effect, is taken together with the well described pro-thrombotic effects of chronic NSAID therapy it raises the question of whether additional aspirin therapy is appropriate. Here I discuss how the data in these studies support a rationale for further investigation of this concept and important considerations for future experimental work in this area.

8.1 The relationship between *in vitro* platelet function and thrombosis *in vivo*

Future work exploring the contribution of aspirin to anti-platelet therapy with $P2Y_{12}$ inhibition requires a strong basis of *in vitro* and mechanistic studies, such as those presented in this thesis. Experiments performed using 96-well plate light transmission aggregometry differ to those performed with traditional LTA, for which there is currently a broader literature. It has, therefore, been important to properly characterise traditional LTA as an assay of platelet function in the presence of both aspirin and strong $P2Y_{12}$ receptor blockade.

The ability to vary the sensitivity of LTA to aspirin and strong $P2Y_{12}$ receptor blockade by varying the physical conditions without changing the apparent characteristics of control responses may explain why this technique is not a clinically useful tool in predicting platelet reactivity [150]. Thus, it is entirely plausible that assay conditions that may appear to show an appropriate response to a specific agonist are inappropriate to measure responses in the presence of pharmacological antagonists, as the mechanism of platelet activation is artificial.

The differences in agonist-specific responses between citrate and lepirudin anti-coagulated samples are more strongly displayed in traditional LTA than in the 96-well plate assay used in this thesis. The amounts of thromboxane A_2 released in citrated samples at various speeds in traditional LTA suggest that the physical forces of higher speed stirring drive the production of TXA_2 and that the production is higher than that seen with the 96-well plate assay. It is possible that these trends hold true in lepirudinised samples, albeit with lower levels of TXA_2 production. Further experiments to delineate the effects of anti-coagulant and stir-speed may be useful for comparison of the two different aggregation assays. It is also important to know if the reversible responses measured are a result of platelet activation and subsequent deactivation or due to unstable adhesive processes. Specifically, experiments should measure the level of integrin $\alpha_{IIb}\beta_3$ activation, as a marker of stable aggregation, and investigate the role of the GPIb/IX/V complex in early, reversible responses in PRP under the conditions previously described.

To understand how the responses measured in these *in vitro* assays relate to *in vivo* thrombus formation I have focused on the responses to collagen, CRP-XL and TRAP-6 amide. Occlusive vascular events are triggered by the exposure of circulating blood to thrombogenic substances. Typically, these substances are collagen and thrombin and their contact with blood is a consequence of atherosclerotic plaque rupture [69]. Whilst plaques may not actually contain thrombin they are rich in tissue factor, which cleaves prothrombin to the active protease [86].

In the *in vitro* and *ex vivo* studies presented here, collagen and thrombin responses have been modeled by collagen and CRP-XL and TRAP-6, respectively. Understanding the responses to these agonists is important in understanding how anti-platelet drugs may prevent occlusive thrombotic events. In the presence of

aspirin and strong $P2Y_{12}$ blockade there is some divergence of responses to these agonists between 96-well plate and traditional light transmission aggregometry. When assessing platelet reactivity by either method the end point of interest is the formation of stable aggregates, which requires the activation of integrin $\alpha_{IIb}\beta_3$ [70, 80].

Mechanisms of collagen-induced aggregation

Collagen-induced platelet aggregation is complex and multiple pathways sense collagen itself and respond to the subsequent soluble mediator release. The two most important collagen receptors are GPVI and the integrin $\alpha_2\beta_1$ [75]. Stimulation of platelets with the specific GPVI agonist, CRP-XL, in traditional LTA results in a large, sustained aggregation response. In lepirudin, this response was abolished in the presence of strong $P2Y_{12}$ inhibition, suggesting that GPVI-mediated stable aggregation requires the actions of ADP at the $P2Y_{12}$ receptor. In fact, the requirement of the ADP-$P2Y_{12}$ axis for responses to GPVI specific stimuli has previously been shown in citrated, aspirin-treated washed platelets [81, 197] and this effect is now confirmed to exist in the presence of plasma, and without the inhibition of cyclo-oxygenase. The measurement of TXB_2, in these samples confirmed that TXA_2 production was not induced by CRP-XL, and this model was not sensitive to aspirin. However, the same experiment in citrated PRP resulted in reasonable production of TXA_2, with aspirin causing a large reduction in stable aggregation. This seems paradoxical as experiments in the absence of TXA_2 suggest that CRP-XL stimulation will lead to downstream $P2Y_{12}$ activity, which would drive aggregation, however this was not the case. The residual activity in the presence of aspirin perhaps represents the actions of ADP. The effect of strong $P2Y_{12}$ blockade was identical to that of aspirin, which is consistent with the effects of TP receptor signaling to cause ADP secretion and, therefore, $P2Y_{12}$ receptor activation. TXA_2 production was also reduced in the presence of strong $P2Y_{12}$ receptor blockade. The

residual aggregation induced presumably reflects an element of TP-receptor activity not associated with the ADP-$P2Y_{12}$ axis. This is not unexpected, as TP-receptor activation in citrated PRP is not entirely inhibited by $P2Y_{12}$ receptor blockade (in traditional LTA). The additive effect of aspirin and strong $P2Y_{12}$ receptor blockade in this model demonstrates that, similarly to lepirudin, GPVI-mediated aggregation is dependent upon soluble mediators.

The understanding of GPVI-induced aggregation can be used to help understand collagen-induced aggregation. Surprisingly, the largely stable collagen-induced aggregation seen in lepirudin is not inhibited by $P2Y_{12}$ receptor blockade, although GPVI-induced aggregation, thought to be a major element of collagen responses [75], is inhibited under these conditions. This confirms that the collagen-induced aggregation in traditional LTA is not solely GPVI-mediated, and likely represents a composite of $\alpha_2\beta_1$ and GPVI activation. Integrin $\alpha_2\beta_1$ exists in two active forms, with either a low or high affinity for collagen, regulated by soluble mediators and GPVI signaling [75, 82]. The high affinity form of $\alpha_2\beta_1$ is reported to promote collagen-GPVI interactions, and is believed to be an important regulator of responses to soluble collagen [75, 83]. This may explain why $P2Y_{12}$ receptor blockade had more limited effects against collagen-induced responses than against CRP-XL in citrated samples, where TXA_2 production was considerable. That said, the final level of stable aggregation in the presence of PAM was only approximately 50% of the initial response after 20 minutes, indicating that early responses were not stable over time. The greater production of TXA_2 and stronger effect of aspirin against collagen-induced aggregation in citrated PRP, than in lepirudin, is consistent with the *in vivo* observation that TP receptor signaling contributes to the $\alpha_2\beta_1$-dependent stabilization of thrombi [198].

The combination of aspirin and strong $P2Y_{12}$ blockade in traditional LTA completely

abrogates any aggregation response, presumably because both $\alpha_2\beta_1$ and GPVI responses are inhibited. In 96-well plate LTA 3µg/ml collagen produced a similarly large response, which was little affected by aspirin, but that was largely inhibited by $P2Y_{12}$ receptor blockade, suggesting that this was a predominantly GPVI-mediated response. This may be due to less interaction between platelets and collagen or because lower levels of TXA_2 production in this system undermine the ability of $\alpha_2\beta_1$ signals to maintain stable interactions with the integrin $\alpha_2\beta_1$.

The exposure of subendothelial or atherosclerotic plaque collagen has been extensively modeled by the shearing of platelet solutions or blood across a collagen coated surface. This interaction is distinct from that of platelets with collagen in a stirred solution, and involves a number of processes, beginning with platelet adhesion/tethering [69]. At shear rates that include the normal arterial range (1000-10000 s^{-1}) [72] platelets bind vWF immobilized onto the surface of collagen via the GPIb/V/IX complex [70, 71], or by the activated integrin $\alpha_{IIb}\beta_3$ [70, 71]. As shear rates increase platelet vWF interactions are predominantly via GPIb/V/IX, although this binding is weak, resulting in platelet rolling across the surface [71]. However, adhesion of platelets via vWF under high shear allows contact between collagen and the GPVI and $\alpha_2\beta_1$ receptors [75]. The role of these receptors varies dependent on the precise formation of the collagen fibres, but in the absence of functioning GPVI thrombus formation does not occur [70] suggesting that this receptor is central to firm adhesion [75]. GPVI binding results in platelet activation and “inside-out” signaling, switching integrins to an active conformation, which provides firm adhesion [82]. The paracrine actions of soluble mediators released from activated platelets promote thrombus growth, potentially resulting in vessel occlusion [69]. The central role of GPVI to thrombus formation on exposed collagen is not modeled in aggregation induced by fibrillar collagen in traditional LTA, based on the results presented in this thesis.

Mechanisms of thrombin-induced aggregation

The modeling of thrombin-induced platelet activation by TRAP-6 is perhaps closer to the *in vivo* activation of this compound as thrombin is a soluble mediator [69, 86]. However, TRAP-6 activates only PAR-1, whereas thrombin activates both PAR-1 and PAR-4 [86].

As for collagen/CRP-XL, there appeared to be a differential activation of platelets by TRAP-6 in either citrated or lepirudinised PRP. Initial responses in lepirudin were not stabilized in the presence of strong $P2Y_{12}$ receptor blockade, suggesting that although early adhesion was induced, integrin $\alpha_{IIb}\beta_3$ activation was ADP-dependent. Like CRP, aspirin had no effect on this response. The differential effect of these agonists in citrate may again be related to increases in TXA_2 production, although this was not measured in these studies, and the effect of aspirin is quite limited. In the 96-well plate assay, strong $P2Y_{12}$ blockade inhibited the responses to equivalent concentrations of TRAP-6, regardless of the anticoagulant used.

For both collagen and TRAP-6, a strong inhibition of aggregation responses was achieved in healthy volunteers after standard dosing of prasugrel, and this was little enhanced by aspirin. Insight into the efficacy of strong $P2Y_{12}$ blockade, with or without aspirin, as an inhibitor of *in vivo* thrombogenesis in response to these agonists can be gained from animal models.

Thrombus formation in vivo

André et al. have shown that platelets from $P2Y_{12}$ deficient mice do not aggregate in response to ADP or low concentrations of either collagen or a mouse-specific TRAP, although *ex vivo* aggregation to high concentrations of both collagen and TRAP are

maintained [199]. Despite this, the induction of arterial thrombosis by $FeCl_3$ is greatly diminished in these mice and is characterized by delayed thrombi formation, increased embolisation and a failure to occlude the injured vessel [199].

The mechanism of *in vivo* thrombus formation is dependent on the injury model used [200]. Ferric chloride-induced injury is believed to be largely collagen-mediated as deficiency of PECAM-1, which inhibits GPVI induced platelet activation [201], accelerates thrombus formation in this model [202] and *in vivo* thrombosis formation was limited in platelets that do not express GPVI [203]. In contrast, laser-induced injury may represent a more thrombin-driven response, as levels of tissue factor are much higher in thrombi in this model than in $FeCl_3$ injured vessels [203].

Measurements of platelet aggregation reported in this thesis suggest that strong $P2Y_{12}$ receptor blockade in man has a similar effect on *ex vivo* platelet aggregation as does $P2Y_{12}$ deficiency [199], and it can be speculated that drugs such as prasugrel and ticagrelor could prevent thrombotic events without the requirement of aspirin [141], although, as explained below, this has not been tested in man. However, the effects of $P2Y_{12}$ receptor blockers alone or in addition to aspirin, have been evaluated in a number of animal models [136, 204-212]. Clopidogrel, at concentrations that strongly inhibit ADP-induced platelet aggregation assessed *ex vivo* by LTA, was effective at blocking cyclic coronary artery flow variations in stenosed canine arteries [204], and after a Folt's crush injury in porcine coronary arteries [205]. Other porcine models have used clopidogrel at concentrations that have varying effects against ADP-induced aggregation by LTA. For instance, Roussi et al. [206] found that using clopidogrel at a dose that reduced ADP-induced aggregation by only 55% largely prevented stent occlusion after crush injury and stenting of a cervical artery. Whilst a further reduction was produced by the addition of aspirin this clopidogrel dose was not associated with strong $P2Y_{12}$ receptor

blockade. Higher concentrations of clopidogrel are effective at reducing thrombus formation in *ex vivo* arterio-venus shunts, with little [207] or no additional effects of aspirin [208]. Murine models of atherosclerotic plaque rupture, in the *Apoe* deficient mouse, show a similar trend for markedly reduced thrombosis with strong $P2Y_{12}$ inhibition [209], and a moderate reduction with partial $P2Y_{12}$ blockade that can be further reduced with aspirin [210].

The novel anti-platelet agent prasugrel reduced thrombus formation in a rat arterio-venus shunt in a dose-dependent manner, with a 60-70% reduction in thrombus weight when animals were treated with 1-3 mg/kg prasugrel [211]. These doses of prasugrel were not tested with aspirin, although the effects of lower doses (0.3-0.6 mg/kg) were enhanced by the addition of aspirin. However, the maximal effect of these combinations was less than that seen with high concentrations of prasugrel alone [136]. A further novel $P2Y_{12}$ antagonist, BX 667, similarly inhibits thrombus formation in a rat arterio-venus shunt, where the percentage inhibition of thrombus formation correlated with the inhibition of ADP-induced platelet aggregation by traditional LTA [212]. This compound also prevented rat carotid artery occlusion after $FeCl_3$ induced injury and inhibited cyclic flow variations after crush injury in canine coronary arteries [212].

Overall, the data from animal models of thrombosis agree with the *in vitro* and *ex vivo* data reported in this thesis: that strong $P2Y_{12}$ blockade has a near maximal effect against platelet reactivity, whereas weaker $P2Y_{12}$ blockade results in a moderate effect that can be increased by the addition of aspirin.

8.2 Anti-platelet therapy

Whilst *in vitro* and pre-clinical *in vivo* models are essential for drug development, drug therapy in patients at risk of thrombotic vascular events should be guided by the results of large, multi-centre clinical trials. Typically these trials test the currently accepted 'best' therapy against an alternative, often novel, therapeutic strategy. In the case of anti-platelet therapy, aspirin is well established as the first-line therapy [125], and so as newer drugs have become available their efficacy has been tested with respect to aspirin and more recently in the presence of aspirin.

For instance, the CAPRIE trial investigated the benefit of $P2Y_{12}$ receptor inhibition by clopidogrel alone, compared to aspirin alone, in those at high risk of ischaemic events (recent manifestations of atherosclerotic disease) – demonstrating that clopidogrel was a superior monotherapy in reducing a predefined end-point of ischaemic stroke, myocardial infarction or vascular death [132]. However, subsequent trials of clopidogrel in patients with vascular disease focused on the addition of clopidogrel to aspirin, a drug combination that improves outcomes over aspirin alone after percutaneous coronary intervention [213, 214], with an optimal effect achieved when clopidogrel therapy is maintained for 12 months post-procedure [214]. The increased anti-thrombotic protection of clopidogrel with aspirin comes at the expense of an increase in adverse bleeding events [214, 215]. This approach was appropriate as clopidogrel was under investigation as an 'add-on', to aspirin and the result is that based on these trials, aspirin plus clopidogrel is now the recommended anti-platelet therapy for 12 months after PCI [216]. The evolution of this treatment protocol reflects the way in which most trials have been designed (to test aspirin with and without clopidogrel), and is not an indication that there is a body of clinical evidence to demonstrate the superiority of aspirin and clopidogrel over clopidogrel alone.

In fact, few trials have investigated the benefits of clopidogrel with and without aspirin, although the MATCH trial reported no significant difference in major vascular events in a high risk population randomised to clopidogrel alone or clopidogrel plus aspirin [217]. This trial did, however, report an increase in life-threatening or major bleeding events in the clopidogrel plus aspirin group compared to clopidogrel alone, the most common being gastrointestinal bleeding events [217].

Although there is a lack of clinical evidence to show that aspirin plus clopidogrel is a more effective therapy than clopidogrel alone I believe that the current clinical data and experimental evidence in chapters 3 and 4 indicates that there is likely to be a large variation in the benefit of additional aspirin, dependent on the level of $P2Y_{12}$ inhibition.

The well-described phenomenon of clopidogrel resistance derives primarily from poor metabolism of clopidogrel to its active metabolite [160], which may be explained by so called 'high-risk pharmacokinetics' [218]. This is a major problem as extensive meta-analysis has shown that residual platelet reactivity whilst on clopidogrel treatment is associated with an increased risk of death and recurrent thrombotic events [219]. Clopidogrel metabolism is cytochrome P450 (CYP) dependent and the function of CYP isoenzymes is affected by both acquired and genetic factors. In particular, the *CYP2C19*2* loss of function allele is associated with decreased clopidogrel active metabolite generation, increased platelet reactivity on clopidogrel and worse outcomes [220-223]. More recently, Bouman et al. reported that the common Q192R polymorphism of the enzyme paraoxonase-1 (PON1) is a crucial determinant of clopidogrel metabolism [129]. Importantly, *PON1* genotype correlated with both the generation of clopidogrel active metabolite and the risk of stent thrombosis, with the QQ192 allele conferring a higher risk [129]. The high incidence

of clopidogrel resistance, which may be greater than 30% [152], indicates that the level of $P2Y_{12}$ inhibition in a generic 'at risk' population is likely to be highly variable [152, 160, 224, 225], which has a major impact on how clinical trial data can be interpreted, particularly for those trials designed to assess the effects of clopidogrel in addition to aspirin. It is reasonable to assume that in the early trials mentioned above that clopidogrel treatment did not produce effective, strong, $P2Y_{12}$ receptor blockade in a portion of the trial participants. This heterogeneous response makes it more likely that a clinical benefit will be seen when aspirin is added to clopidogrel, i.e. some patients have little anti-thrombotic protection when receiving clopidogrel alone and so the level of anti-thrombotic protection is enhanced by the addition of aspirin. Conversely, there will be patients who benefit fully from clopidogrel, i.e. they have a high level of $P2Y_{12}$ inhibition on therapy, and it is less clear whether or not these patients benefit from the inclusion of aspirin, although the risk of adverse bleeding events will be increased.

Non-responsiveness to clopidogrel can be identified by a number of techniques, the recognized 'gold standard' of which is the flow cytometric analysis of platelet VASP phosphorylation – a marker of adenylyl cyclase activity and intra-platelet cAMP levels [152, 226]. The VerifyNow™ point-of-care assay for $P2Y_{12}$ is also a validated tool to identify clopidogrel resistance [227-230]. Whilst clopidogrel non-responders can be readily identified with appropriate platelet function testing, attempts to overcome clopidogrel resistance pharmacologically, by increasing the standard daily dose two-fold, are ineffective as even at a double-dose of clopidogrel there is likely to be a resistant sub-population [231]. In plain terms, it is not possible to achieve strong $P2Y_{12}$ receptor blockade across a generic 'at risk' population with clopidogrel [231].

The *in vitro* and *in vivo* work presented in chapters 3 and 5 demonstrates that with the novel $P2Y_{12}$ receptor blocker prasugrel it is possible to achieve a strong level of

$P2Y_{12}$ inhibition. Although the active metabolites of prasugrel and clopidogrel are structurally and functionally very similar, prasugrel is more effective than clopidogrel as a dual anti-platelet therapy with aspirin [135]. This may be because prasugrel is oxidised to its active metabolite R-138727 in two steps, although only one of these is cytochrome P450 dependent. This reduces the opportunity for both genetic and acquired resistance [232] resulting in more homogenous responses to treatment in at risk populations. Similarly, the orally active $P2Y_{12}$ receptor blocker ticagrelor, when used with aspirin, provides a significantly better anti-thrombotic effect than clopidogrel and aspirin [140].

Whilst the superior effects of prasugrel and ticagrelor, over clopidogrel, may be due to a more consistent level of $P2Y_{12}$ inhibition across the study populations as a whole, there is also evidence to suggest that other pharmacodynamic effects contribute to the increased anti-thrombotic effect, independent of enhanced active metabolite formation [134, 222, 233]. Additionally, ticagrelor and aspirin as a dual anti-platelet therapy has the unique effect of reducing mortality from any cause in the 12 months following an acute coronary syndrome event in comparison to clopidogrel and aspirin [140]. The true cause of this effect is unknown, although it has been attributed to a secondary increase in circulating adenosine associated with the actions of ticagrelor in the blood, and may be mediated by adenosine-induced coronary vasodilatation [112, 234] or by enhancement of the anti-aggregatory effect of adenosine [111]. Elevated plasma adenosine is also attributed as the cause of increased dyspnoea among patients taking ticagrelor [235].

8.3 The adverse and extra-platelet effects of aspirin and other COX inhibitors

Unfortunately, the benefits of anti-platelet therapy come at the cost of an increase in adverse bleeding events, as haemostatic mechanisms are disrupted. Whilst aspirin alone is associated with an increased risk of bleeding [29], this is furthered when other anti-platelet agents are added [142], and is likely to reflect the total level of platelet inhibition. For instance, the bleeding risk of prasugrel, clopidogrel and ticlopidine, in a pre-clinical model, correlated with the anti-platelet and anti-thrombotic effect of each compound [211]. As a NSAID, aspirin is also associated with an increased risk of gastrointestinal (GI) erosions and ulcerations, which can result in severe bleeding events [29, 36]. Combined with its effects on platelets aspirin therefore has a dual potential to cause adverse bleeding events.

Meta-analysis of randomized controlled trials where aspirin has been used for primary and secondary prevention indicates the risk of unwanted bleeding in patients taking aspirin [127]. In primary prevention the relative risk of extracranial bleeding, which is predominantly GI bleeding, is 1.54 (1.30-1.82) in those taking aspirin compared to controls [127]. A similar trend is seen in patients using aspirin for secondary prevention of thrombotic events, with a potentially higher relative risk (2.69; 1.25-5.76), although these data are based on relatively few studies that have recorded this outcome [127]. It is suggested that at lower aspirin doses this represents a non-specific anti-platelet effect, resulting in reduced resolution of small gastric lesions, with an increasing role of GI COX inhibition with higher doses [29].

Whilst the relative risks described above are from carefully controlled conditions, these are generally not the conditions encountered across a global population. Collection of patient registry data allows interrogation of large patient cohorts to

answer specific clinical questions. This approach has been used to assess the association between serious bleeding events and vascular events and outcomes [236]. An international, prospective observational study was performed on 1-year follow up data from 64 977 patients with prior ischaemic vascular events or multiple risk factors for atherosclerosis. Within this population, 1.00% of patients with symptomatic vascular disease suffered a serious bleeding event within 1 year of follow up, although in those with disease risk factors only the rate was lower (0.59%) [236]. Interestingly, patients who suffered serious bleeding events were more likely to suffer a thrombotic vascular event (MI or stroke), and had a higher mortality rate then those who did not (Hazard Ratio ≥ 2) [236]. Furthermore, patients with serious bleeds were more likely to discontinue aspirin use over the study period, and whilst there are no data to temporally associate bleeding events, discontinuation of aspirin and thrombotic events it is suggested that this progressive sequence of events may occur [236]. In fact, a systematic review and meta-analysis addressing the risks of discontinuing, or not adhering to, aspirin therapy in patients at risk for coronary artery disease identified an increased risk of thrombotic events after aspirin withdrawal [237]. However, this review drew data primarily from observational studies with varied clinical endpoints and study designs [237]. Clearly further prospective studies are required to fully understand the associations between bleeding events and the risk of vascular events.

GI damage by NSAIDs is partly due to the inhibition of COX-1 [45] resulting in the loss of PGE_2 and PGI_2, which normally act as a brake on acid secretion and stimulate (protective) mucus production [36]. Therefore, it is recommended therapy to include a proton pump inhibitor (PPI) in combination with aspirin therapy for patients with a history of GI bleeding [216, 238]. In the case of patients who have major GI bleeds whilst taking aspirin for protection against cardiovascular risk, it is recommended therapy to switch to clopidogrel alone [216].

Whilst proton pump inhibitors are effective at reducing upper gastrointestinal bleeding there has been some concern that they may interact with clopidogrel, potentially reducing its efficacy [239]. This hypothesis is based on observational data, associating concomitant proton pump inhibitors and clopidogrel use with higher cardiovascular mortality than clopidogrel alone and that both proton pump inhibitors and clopidogrel may utilise the CYP2C19 metabolic pathway [239]. Although a randomized placebo-controlled crossover study in healthy volunteers showed that some interaction exists between clopidogrel and omeprazole, resulting in decreased efficacy of clopidogrel [240], these results do not relate to a clinical effect [241]. In a randomized controlled clinical outcome study the use of omeprazole with clopidogrel reduced the rate of upper GI bleeding without changing the cardiovascular effect, although this does not necessarily reflect a class effect of PPIs [241].

Given the extra-platelet effects of aspirin that may potentiate adverse bleeding events it is important to determine whether aspirin does actually improve the anti-platelet effect achieved with strong $P2Y_{12}$ inhibition alone. Certainly the *in vitro* work shown here with PAM and ticagrelor, and the *in vivo* work with prasugrel suggests that the risks and benefits of including aspirin with these novel therapies should be further investigated.

As aspirin has the ability to inhibit COX activity globally, the extra-platelet effects of aspirin are certainly not limited to the gut. In fact, perhaps more important in cardiovascular disease are the effects of aspirin on the vasculature, and in particular, the vascular endothelium [7, 9]. Endothelial COX is required for the generation of prostacyclin, which is important as both an anti-aggregatory and vasodilating hormone. The activity of endothelial COX is often assessed by the presence of the urinary PGI_2 metabolite, PGI-M [7, 49]. Data from healthy volunteers, presented in

chapter 5, indicates that low dose aspirin can inhibit this marker of endothelial function, whilst contributing little to the overall anti-platelet effect. Although healthy endothelium has the ability to overcome irreversible COX inhibition by *de novo* enzyme synthesis, this is not thought to occur as others have shown that the reduction of urinary PGI-M persists for up to 24 hours after the cessation of once daily dosing for the previous seven days [242]. This is important, as loss of vascular prostacyclin is the presumptive mechanism for the increased risk of thrombotic vascular events observed in patients taking NSAIDs [49].

The anti-aggregatory effects of prostacyclin are mediated through the platelet IP receptor, a G_s-protein coupled receptor that activates adenylyl cyclase and ultimately signals an increase in intraplatelet cAMP. This is the opposite effect of $P2Y_{12}$ receptor agonism, which inhibits adenylyl cyclase and results in decreased cAMP [243]. When the $P2Y_{12}$ receptor is inhibited, the sensitivity of the IP receptor signaling pathway is increased, and this may further the anti-platelet effect that can be achieved with strong $P2Y_{12}$ receptor blockade [107]. Clearly, loss of prostacyclin does not allow this process to occur and so the concomitant use of aspirin may limit the anti-platelet effect of $P2Y_{12}$ receptor blockers.

In cardiovascular disease loss of PGI_2 may be particularly relevant as PGI_2 is involved in maintaining normal endothelial function through its actions on vascular IP receptors and PPARβ [244, 245]. In atherosclerosis, PGI_2 production is increased [154, 246] and is reported to act as an anti-atherogenic factor by inhibiting platelet activation and leukocyte-endothelial interactions [247]. Loss of PGI_2 is therefore considered to be both pro-atherogenic and pro-atherothrombotic and this is supported by the accelerated progression of atherosclerosis in patients with a variant prostacyclin receptor that does not activate adenylyl cyclase [248].

It is often stated that low dose aspirin exerts its effects by preferential inhibition of platelet COX-1 whilst sparing endothelial COX, which some believe is predominantly COX-2 [29]. However, low dose aspirin consistently inhibits both platelet and endothelial COX, reducing urinary TX-M and PGI-M [53]. Although the cardiotoxicity of NSAIDs is not defined by COX-2 selectivity [54, 179], it is an adverse effect associated with NSAID doses that inhibit COX-2 to achieve their therapeutic benefit. Despite this, aspirin does provide secondary prevention against thrombotic events, and higher doses of aspirin as a monotherapy are not associated with a decreased efficacy, only an increase in adverse bleeding events [36, 249].

It can be argued that the effect of aspirin on *in vivo* TXA_2 production is sufficient to produce a net anti-thrombotic effect despite the loss of protective prostacyclin, and when aspirin is used with clopidogrel the risk of thrombosis is further reduced, although it is still rational to assume that the accompanying loss of prostacyclin limits the anti-thrombotic effect [141]. With strong $P2Y_{12}$ receptor blockade by prasugrel alone, as has been shown in this thesis, there is a strong anti-platelet effect and levels of prostacyclin metabolites are not reduced.

Strong $P2Y_{12}$ receptor blockade alone has not been compared, in clinical trials, to either aspirin alone or aspirin in combination with a strong $P2Y_{12}$ inhibitor and so there is currently no clinical data to illuminate this hypothesis. There is however, an anecdotal suggestion that higher doses of aspirin in combination with a strong $P2Y_{12}$ inhibitor may be less effective than lower aspirin doses in a dual therapy [21]. The PLATO randomized-controlled trial, conducted at multiple centers in 4 pre-defined geographic regions, compared the efficacy of ticagrelor to clopidogrel (both with aspirin) at reducing the composite rate of cardiovascular death, MI or stroke after an ACS event. Overall, ticagrelor was superior to clopidogrel, although in the North American region the primary endpoint was reached more often in those randomized

to ticagrelor. This effect was attributed to differences in responses within the United States, where the Hazard Ratio for endpoint events was 1.27, compared to 0.84 globally [140]. In applying for US FDA approval to market ticagrelor the patent owners and trial sponsors, AstraZeneca, indicated that *post hoc* multivariate analyses suggested that this phenomenon was associated with higher aspirin doses used in the US region, compared to other trial centres. In fact, the aspirin dose in the US was dichotomized between 85mg and 325mg daily, with a tendency towards higher endpoint events in the high dose aspirin group [250]. However, FDA advisory panel did not accept this explanation, suggesting it was confounded by differences in baseline characteristics and treatment between those taking low and high dose aspirin [251].

8.4 Conclusions

In this thesis I have aimed to understand the interactions between the platelet cyclo-oxygenase and $P2Y_{12}$ pathways as targets for anti-thrombotic therapy. Although aspirin is an effective anti-thrombotic agent it is apparent that a large proportion of its anti-platelet effects can also be achieved with strong $P2Y_{12}$ receptor blockade alone. Whether or not aspirin enhances the anti-aggregatory effects of strong $P2Y_{12}$ receptor blockade is still unclear. In this thesis I have presented *in vitro* and *ex vivo* data suggesting that aspirin offers little enhancement to the effects of prasugrel (or its active metabolite). However, I have also demonstrated that this conclusion is dependent upon specific assay conditions, varying of which can lead to markedly different results. Clearly, future work in this field will require careful selection of appropriate *in vitro* tests

Given the established toxicity of aspirin in the gastrointestinal system and its potential to cause cardiovascular toxicity, this thesis contributes a clear rationale for

future work to investigate the role of this drug alongside strong $P2Y_{12}$ receptor blockers in the secondary prevention of thrombotic vascular events. There is also further work required to assess the roles of COX-1 and COX-2 in supporting the production of PGI_2, in particular, within the cardiovascular system. In particular, there is still the unexplained mismatch between the effects of aspirin, NSAIDs and COX-2-selective drugs on urinary PGI_2 metabolites and the distributions of COX isoforms within the circulation.

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