
The Regulation of Human PAI-1 Levels in Blood

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by

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Abstract

Plasminogen activator inhibitor type 1 (PAI-1) is the major inhibitor of fibrinolysis. Plasma PAI-1 levels show clear circadian oscillation, peaking in the morning. Expression of the PAI-1 gene may be directly influenced by the -675 4G/5G polymorphism in the PAI-1 promoter. The aim of the present study was to investigate the regulation of PAI-1, using monocytes and platelets as cell models, under conditions that would simulate events occurring within a forming thrombus.

Blood from donors genotyped for the 4G/5G polymorphism was used. Direct stimulation of monocyte with lipopolysaccharide (LPS), induced a small increase in *Pai-1* expression (710-fold) above baseline, however, monocyte *Pai-1* expression was markedly increased (4700-fold) by interaction with platelets activated by cross-linked collagen related peptide (XL-CRP). Furthermore, the induction of monocyte PAI-1 expression by platelets was not dependent upon cell-cell contact, but appeared to be mediated by both exogenous and endogenous release of TGF- β 1. The induction of monocyte *Pai-1* expression by either LPS or CRP-XL was not associated with the 4G/5G polymorphism, but the amount of PAI-1 released into plasma following stimulation was influenced by the polymorphism.

Five peripheral clock genes examined were expressed in monocytes under resting conditions. LPS induced the expression of *Dec1* and *Bmal2* but rapidly switched off the expression of *mPer2* and *clock* in monocytes, whereas the stimulation of monocytes by CRP-XL did not affect the expression of clock genes, apart from the down-regulation of *Clock* and up-regulation of *mPer2*. Therefore, the acute induction of PAI-1 in blood cells does not appear to be dependent on the circadian clock.

Platelet count significantly increased during the afternoon compared to the morning. But platelet *Pai-1* mRNA abundance did not exhibit significant diurnal variation nor was it influenced by the 4G/5G polymorphism. Although anucleate, platelets appear to retain the ability to synthesise PAI-1, which could be partially inhibited by cycloheximide, both the synthesis of PAI-1, and release from α -granules of activated platelets, was related to the 4G/5G polymorphism, with the highest level of PAI-1 synthesised and released by platelets from 4G homozygous subjects.

In conclusion, PAI-1 in circulatory cells is regulated by circadian factors under resting conditions, but circadian influences may be masked following stimulation. The 4G/5G polymorphism regulates the amount of PAI-1 released by both stimulated monocytes and platelets, and may therefore play a role in the formation or resolution of a thrombus.

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List of Abbreviations

ACE	Angiotensin converting enzyme
ADP	Adenosine diphosphate
AngII	AngiotensinII
ANOVA	Analysis of variance
AT	Antithrombin
AT1	AngiotensinII type 1 receptor
BAECs	Bovine aortic endothelial cells
Bcl3	B cell lymphoma 3
bHLH	Basic helix-loop-helix
bHLHZIP	Basic helix-loop-helix/leucine zipper
CAD	Coronary artery disease
CAT	Chloramphenicol acetyltransferase
CFT	Clot formation time
ChIP	Chromatin immunoprecipitation assay
COX-1	Cyclo-oxygenase-1
COX-2	Cyclo-oxygenase-2
CRP-XL	Cross-linked collagen-related peptide
CRY	Cryptochrome
CT	Clotting time
Ct	Cycle threshold
CVD	Cardiovascular disease
DEC	Differentially Expressed in Chondrocytes
ECLT	Euglobulin clot lysis time
ELISA	Enzyme-linked Immunosorbant assay
EMSA	Electromobility shift assay
ERE	Estrogen response element
ER α	Estrogen receptor alpha
GPIX	Glycoprotein-IX
GPVI	Glycoprotein-VI
GRE	Glucocorticoid response element
HRE	Hypoxia-response element
HUVEC	Endothelial cells
IL-1	Interleukin-1
ITP	Idiopathic thrombocytopenic purpura
LPS	Lipopolysaccharide
MCF	Maximal clot firmness
MI	Myocardial infarction
miRNA	microRNA
MNCs	Human mononuclear cells

MPC	Magnetic Particle Concentrator
PA	Plasminogen activators
PAI-1	Plasminogen activator inhibitor type-1
PAS	PER-ARNT-SIM
PBS	Phosphate buffered saline
PER	Period
PGE-2	Prostaglandin E2
PKA	Protein kinase A
PNACL	Protein and Nucleic Acid Chemistry Laboratory
PPP	Platelet poor plasma
PRP	Platelet-rich plasma
PSGL-1	P-selectin glycoprotein ligand-1
RAS	Rennin-angiotensin system
REV-ERB α	Nuclear receptor reverse strand c-ERB α
ROR	Retinoid-related Orphan Receptor
RPE	R-phycoerythrin
rP-Selectin	Recombinant P-selectin
rTGF- β 1	Recombinant TGF- β 1
SBE	Smad binding element
SCN	Suprachiasmatic nucleus
TEG	Thromboelastography
TEM	Thromboelastometry
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGF- β	Transforming growth factor-beta
TLR4	Toll-like receptor 4
TNF α	Tumor necrosis factor-alpha
tPA	Tissue-type plasminogen activator
TRAP	Thrombin receptor-activating peptide
TSP-1	Thrombospondin-1
TVO	Thrombotic vascular occlusion
uPA	Urokinase-type plasminogen activator
USF	Upstream stimulating factor
VLDLRE	Very-low-density lipoprotein response element
Vn	Vitronectin

Chapter 1

Introduction

1.1 –Cardiovascular disease

Cardiovascular disease (CVD) is the main cause of death worldwide and in the UK alone, diseases of the heart and arteries account for just under 238,000 deaths a year (around 39% of all deaths; reviewed in Petersen *et al.* 2004), which is more than all forms of cancer combined. Coronary artery disease (CAD) is a common form of CVD, and it is disease of the arteries that supply the myocardium with blood. The most common cause of CAD is atherosclerosis. Atherosclerosis is a chronic and progressive disease of the arteries, and in the advanced stages of the disease process, atherosclerotic plaque develop. At first, the cross-sectional area of the lumen will be maintained through compensatory remodeling by the coronary arteries (Glagov *et al.* 1987). The progression of the disease might therefore be clinically silent for years. However, in the long run, these plaques cause narrowing of the lumen of the coronary arteries, making the blood flow through these arteries more difficult. In addition, disruption of the atherosclerotic plaque can occur at any time, resulting in thrombosis. The production of the thrombi from ruptured plaques can cause total occlusion of the coronary artery causing damage to the myocardium, termed heart attack or myocardial infarction (MI). The occlusive thrombus also causes ischemia, and the longer term effects of ischemia, such as necrosis can lead to heart failure over time.

The frequency of atherothrombotic events, including MI, ischemic stroke and pulmonary thromboembolism displays significant circadian variation with peak incidence in the early morning, between 6 AM and 12 noon (Muller *et al.* 1985; Tsementzis *et al.* 1985; Colantonio *et al.* 1989). This morning excess in cardiovascular

events may result in part from antiphase circadian variation in fibrinolytic activity, resulting from circadian oscillations of various factors including plasminogen activator inhibitor type-1 (PAI-1).

1.2 – PAI-1 in thrombosis and fibrinolysis

PAI-1 is an inhibitor of plasminogen activation, and is considered to be an important regulator of fibrinolysis. The mechanisms of the coagulation/fibrinolytic system are complex and interlinked. The extrinsic coagulation pathway is initiated when blood is exposed to cell-bound tissue factor (TF). TF binds to activated factor VII (FVIIa), and the TF-activated factor VIIa complex activates factor X and IX to form Xa and IXa. Factor IXa in turn activates additional factor X. Factor Xa converts prothrombin (FII) to thrombin (FIIa). Thrombin activates platelets through protease-activated receptors 1 and 4, and also cleaves fibrinogen to generate fibrin monomers, which then polymerise into stable cross-linked fibrin. Once stable cross-linked fibrin is established, the fibrinolytic system can act.

Fibrinolysis is dependent on the balance between plasminogen activators (PA) [urokinase-type PA (uPA) and tissue-type PA (tPA)], and PAI-1, which is the major physiological inhibitor of PA in plasma (Saksela and Rifkin 1998). Plasminogen is converted by PAs to the active enzyme, plasmin, which is a protease that lyses fibrin clots. One important mechanism for regulating plasmin production involves the formation of complexes between PAI-1 and PAs, which prevents the conversion of plasminogen to plasmin (Sprengers and Kluft 1987).

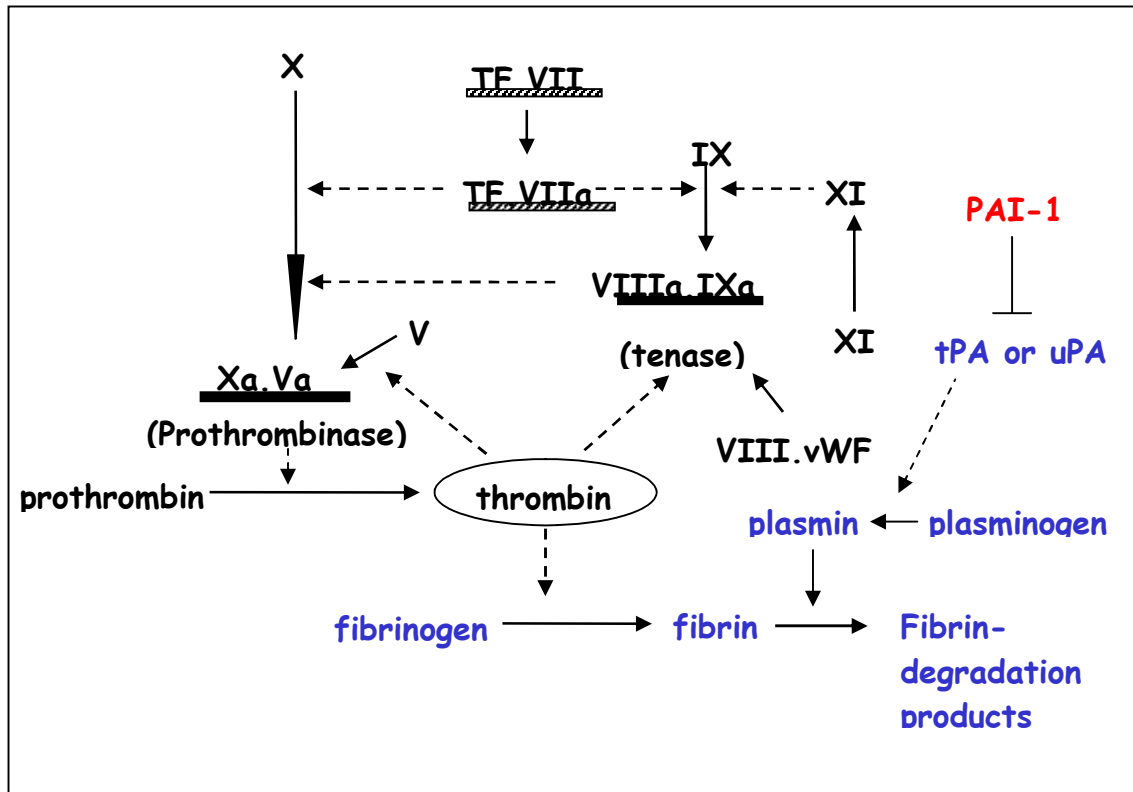


Figure 1.1 – Diagram of the coagulation and fibrinolytic system

Coagulation (depicted in black) is initiated by contact between plasma-borne clotting factors and tissue factor (TF or thromboplastin). TF binds to activated FVII (FVIIa) and this complex activates FX to FXa. TF also activates FIX to FIXa which, with the cofactor FVIIIa (known as the tenase complex) increases the rate of conversion of FX to FXa. FXa binds to the cofactor FVa on a negatively charged lipid surface. The complex, known as the prothrombinase complex, activated prothrombin (FII) to thrombin (FIIa). Thrombin cleaves fibrinogen into fibrin. The fibrin molecules cross-link to form a clot. The clearance of the fibrin clot (termed fibrinolysis, depicted in blue) is carried out by the protease plasmin, which is converted from plasminogen through the action of plasminogen activators (PA). Fibrinolysis is attenuated by a number of inhibitors including PAI-1, which acts by binding to PA.

Introduction

Under physiological conditions, plasminogen, t-PA, and fibrin form a ternary complex that promotes the development of plasmin and the subsequent lysis of fibrin. This allows plasmin and tPA to be protected from inactivation by their respective inhibitors. However, PAI-1 is still able to suppress both tPA and uPA as it can also bind to fibrin. The binding of tPA to fibrin may in part explain the inhibitory effect of PAI-1 on fibrinolysis. The addition of polyclonal antibodies against PAI-1 in a developing thrombus increases the rate of fibrinolysis and reduces the extension of the clot, suggesting that PAI-1 promotes the stability and extension of the thrombus (Chandler *et al.* 1958). After it has been activated in platelets, PAI-1 is fixed within the clot by binding to fibrin and retains its capacity to inhibit uPA and tPA, increasing the thrombus resistance to lysis. In the circulation, most tPA is bound to PAI-1, but also a small proportion is either free or bound to fibrin.

Under pathological conditions, a decrease in fibrinolysis due to high plasma PAI-1 concentrations might be expected to result in a decrease in thrombolysis rate, and therefore increase the rate of thrombus formation. High plasma PAI-1 concentrations are indeed associated with various thrombotic disorders and disease states. Numerous studies have demonstrated a relationship between plasma PAI-1 levels and thrombotic status. Increased levels of PAI-1 have a robust correlation with increased risk of MI and ischemic stroke (Held *et al.* 1997; Thogersen *et al.* 1998; Johansson *et al.* 2000). PAI-1 appears to be the major determinant of tPA activity in the blood because it has the highest affinity for tPA and is the only inhibitor found complexed with tPA in plasma (reviewed in Kruithof 1988). Current data suggest that substantial increases in tissue PAI-1 expression contribute to increased thrombus formation and vascular injury (reviewed in Vaughan 2005).

1.3 – Circadian variations in fibrinolysis

During basal conditions, tPA is produced in a circadian rhythmic pattern (Andreotti *et al.* 1988; 1991), and PAI-1 also displays circadian oscillations in the plasma, thus fibrinolytic activity should display circadian variation. Indeed clinically, the circadian rhythm in fibrinolysis has been shown for a long time (Fearnley *et al.* 1957). Rosing *et al.* (1970) reported a circadian rhythm in global fibrinolytic activity examined by the fibrin plate assay in blood samples obtained from clinically healthy, diurnally active, non-fasting subjects, with peak fibrinolytic activity measured in the afternoon. Activation of fibrinolysis was also demonstrated by the euglobulin clot lysis time (ECLT) when subjects assumed an upright position. The extent of this activation response varies in a circadian manner, with maximum effect in the morning and minimum effect in the afternoon (Kofoed *et al.* 1994). Multiple exogenous factors that activate fibrinolysis interact with or mask the expression of this endogenous circadian rhythm. For example, increase in fibrinolysis is observed after physical exercise, the increase being highest in the afternoon (Rosing *et al.* 1970).

The relative hypercoagulability as a result of the large amplitude of the circadian rhythm in fibrinolysis may also lead to a relative hypocoagulability of the blood in the evening and early night, which is predisposing for hemorrhagic accidents. In patients with cirrhosis of the liver and esophageal varices, variceal bleeding displays a circadian rhythm with two peaks, a major one in the evening between 7 and 11 pm and one minor one in the morning, between 7 and 9 am (Garcia-Pagán *et al.* 1994; Siringo *et al.* 1996; Mann *et al.* 1999). Piscaglia *et al.* (2000 and 2002) found patients with cirrhosis of the

liver to be in a hyperfibrinolytic state, but still demonstrating a circadian rhythm in fibrinolysis, with the timing of peak and trough activity being comparable to that of clinically healthy subjects. The peak in total fibrinolytic activity and in tPA activity in the cirrhotic patients occurred between 6 and 10 pm, respectively, corresponding to the major peak in the occurrence of variceal haemorrhages.

1.4 – Structure of PAI-1

PAI-1, a member of the serpin superfamily, is a single-chain glycoprotein with a molecular weight of 47,000 daltons. It consists of 379 amino acids and a 23 amino acid signal peptide indicating that it is a secreted protein. It lacks cysteine but contains multiple methionines (Gils *et al.* 2003). PAI-1 is synthesised and secreted in an active form, but it can decay to a “latent form” through conformational changes that mask the reactive site, making it inactive as an inhibitor. This instability of active PAI-1 maybe due to the lack of cysteine residues, and although the biosynthesis rate is high, this property of PAI-1 produces a short biological half life of only 8-10 minutes, which yields a relatively low plasma concentration of effective PAI-1 (about 20ng/ml; 400pM). In plasma, PAI-1 is stabilised in the active conformation by binding to vitronectin (Vn), which prolongs its activity by >10 times (Declerck *et al.* 1988).

1.5 – Cellular Origin of PAI-1

PAI-1 was first isolated from rabbit and bovine endothelial cells (Loskutoff *et al.* 1983), and rat hepatoma cells (Coleman *et al.* 1982). Subsequent studies demonstrate

endothelial cells, hepatocytes, adipocytes and platelets are the major source of plasma PAI-1 (Declerck *et al.* 1988; Chomiki *et al.* 1994; Samad and Loskutoff 1996). Endothelial cells in culture synthesize PAI-1 at relatively high rates (Schleef and Loskutoff 1988), but only under stimulation. Cultured human hepatocytes, on the other hand, constitutively secrete PAI-1 (Busso *et al.* 1994), and furthermore, normal human liver cells have been demonstrated to express *Pai-1* (Chomiki *et al.* 1994). Hence, hepatocyte and not the vascular endothelium is likely to be a major contributor to circulating PAI-1 levels.

PAI-1 is found in the alpha-granules of platelets and is released upon activation and degranulation. The patho-physiologic importance of the platelet PAI-1 pool for inhibition of the fibrinolytic system has been difficult to reconcile with the fact that the majority of PAI-1 in platelets exists in a predominantly inactive or latent form. Previous studies have shown that only 5% to 10% of the PAI-1 present in the platelets is in an active configuration that could complex-bind and thereby inhibit tPA (Schleef *et al.* 1985; Booth *et al.* 1988). However, other reports suggest that PAI-1 release by platelets following activation binds to Vn in the fibrin clot and become active. Morgenstern *et al.* (2001) demonstrated that protein kinase A (PKA), Vn and PAI-1 translocated onto the fibrin fibers following platelet activation. Together with the group's finding that PKA phosphorylation of Vn reduces its grip of PAI-1, this suggests that platelets can release PAI-1 in its active state. In addition Podor's group (Podor *et al.* 2002), using confocal microscopy, found that Vn:PAI-1 complexes produced by activated platelets co-localize with platelet vimentin cytoskeleton on the surface of platelets in platelet rich plasma

clots. These findings suggest Vn may play a role in the regulation of platelet PAI-1 activity.

Although platelets lack nuclear DNA they retain mRNA from the megakaryocyte, (Booyse and Rafelson 1967a; Newman *et al.* 1988). It has been shown that platelets retain the ability for protein synthesis and can synthesize at least some proteins (Warshaw *et al.* 1967; Kieffer *et al.* 1987). Work by Brogren and colleagues (2004), have provided direct experimental evidence that platelets contain significant amounts of PAI-1 mRNA that is translationally active. Because the major part of the newly synthesized PAI-1 was found to be active, the relative PAI-1 content in platelet rich clots could influence their resistance to thrombolysis (Potter van Loon *et al.* 1992). This hypothesis, that PAI-1 released from platelets is an important determinant of thrombolysis resistance, is supported by *in vitro* clot lysis studies on platelets from a patient with complete loss of PAI-1 expression (Fay *et al.* 1994), as well as studies on thrombus generated in the Chandler loop (Stringer *et al.* 1994).

Under pathological conditions, however, several other cell types secrete a fairly large amount of PAI-1, including monocytes (Hamilton *et al.* 1993a; Lundgren *et al.* 1994). The monocyte has the ability to affect both coagulation and fibrinolytic systems, as peripheral blood monocytes can express PAI-1. It has been showed that blockade of the renin–angiotensin system by an angiotensin-converting enzyme inhibitor reduces the synthesis of PAI-1 in cultured human monocytes (Nagata *et al.* 2001). Thus, controlling PAI-1 production by monocytes and macrophages may be a target for treating coronary artery disease.

It has been proposed that adipose tissue may contribute directly to the elevated PAI-1 levels in obesity (Alessi *et al.* 1997; Loskutoff and Samad 1998). The expression of PAI-1 has been demonstrated in cultured adipose cell line (Lundgren *et al.* 1996) and in adipose tissue from rodents (Samad *et al.* 1996; Loskutoff and Samad 1998) and humans (Alessi *et al.* 1997; Eriksson *et al.* 1998a). In addition, it was demonstrated that adipose tissue PAI-1 gene expression and release of PAI-1 were increased in obesity (Eriksson *et al.* 1998a).

1.6 – Regulation of PAI-1

Regulation of plasma PAI-1 has been well studied, and appears to be a complicated process. This thesis will focus on the genetic determinants of plasma PAI-1, its regulation by the peripheral clock, and regulatory factors that have been identified to play a role in PAI-1 transcription.

1.6.1 – PAI-1 Gene Polymorphism and association with CVD

Rallidis's group has demonstrated that increased PAI-1 levels are seen in the offspring of men who experienced MI before the age of 55 (Rallidis *et al.* 1996). This finding supports the hypothesis of a familial predisposition to coronary artery disease, based on a defective fibrinolytic system. Indeed, PAI-1 levels partly depend on genetic factors. Three polymorphisms in the PAI-1 gene seem to associate with different levels of PAI-1: firstly an eight-allele (CA)_n repeat polymorphism in intron 3 (Dawson *et al.* 1991), and secondly a two-allele HindIII restriction fragment length polymorphism of the 3'

flanking region (Dawson *et al.* 1993). An additional polymorphism, situated 675-bp upstream of the transcription start site, contains a single guanine insertion/deletion variation appropriately termed *4G* or *5G* (Fig. 1.2). Individuals homozygous for the *4G* allele shows the highest PAI-1 plasma levels (Margaglione *et al.* 1998), and also have higher levels of PAI-1 activity and antigen in platelets, indicating a possible increased resistance against endogenous and external plasminogen activation (Nordenhem and Wiman 1997). *In vitro* studies have recognised differential binding of transcription regulating proteins at this site. Elevated gene transcription is associated with four guanine bases (the *4G* allele), and in the binding of a common transcriptional activator alone, whereas DNA with five guanine bases (the *5G* allele), there is also binding of an as yet unidentified protein that decreases the binding of the activator (Dawson *et al.* 1993; Eriksson *et al.* 1995). The *4G/5G* promoter polymorphism has been shown to be related not only to increased PAI-1 levels in healthy individuals but also in patients with arterial and venous thromboembolic disorders (Eriksson *et al.* 1995; Ossei-Gerning *et al.* 1997). However, conflicting data exists in the association of *4G/5G* polymorphism with an increased risk of cardiovascular diseases such as CAD and stroke.

In a large meta-analysis of 37 studies, Ye *et al.* (2006) demonstrated that the *4G* variant of the PAI-1 gene has relatively little effect on coronary disease with per-allele relative risk of 1.06 (1.02-1.10). In another meta-analysis of 9 mainly case-control studies a modestly increased risk of myocardial infarction was observed for the *4G* homozygous genotype with per-allele relative risk of 1.20 (1.04-1.39) (Boekholdt *et al.* 2001). In another study conducted in Japan, 112 polymorphisms were studied simultaneously, and concluded that the *4G/5G* polymorphism was one of the two polymorphisms that were

associated with MI in women (Yamada *et al.* 2002). However in a large case-control study the 4G/5G polymorphism was not associated with risk of an acute MI at young age (Italian Study Group 2003). Iwai's group demonstrated that the 4G/5G polymorphism was associated with a faster progression to acute coronary syndrome after first anginal pain (Iwai *et al.* 1998). In postmenopausal women (Roest and Banga 2000) and in the elderly (Heijmans *et al.* 1999), the 4G/5G polymorphism was not predictive for fatal MI. In another Study, the 4G/5G polymorphism was not predictive for future MI in men initially free of cardiovascular disease (Ridker *et al.* 1997).

In stroke patients, increased PAI-1 levels have been shown compared to healthy controls, both in the acute-phase, and even several months after the event (Margaglione *et al.* 1994; Lindgren *et al.* 1996; Catto *et al.* 1997). However, Johansson's group (2000) found that plasma PAI-1 levels were not significantly associated with increased incidence of stroke in a population-based cohort. A tendency for a protective effect of the PAI-1 4G homozygous genotype in stroke has been demonstrated in various studies (Roest and Banga 2003; Hoekstra *et al.* 2003), although in only one study was the effect statistically significant (Roest *et al.* 2000). The involvement of PAI-1 in stroke appears to be more complicated, as a case-control study carried out in Korea, demonstrated that the 4G allele was associated with an increased risk of ischemic stroke (Band *et al.* 2001). The genetic background of the study population may possibly explain this inconsistent finding. A protective role of the 4G allele in stroke, as opposed to an increased in risk of MI, may indicate a difference in pathogenesis of these diseases.

1.6.2 – Circadian clock

Plasma PAI-1 demonstrates a circadian pattern with peak levels observed in the morning. To appreciate the molecular mechanism behind the regulation of PAI-1 by the circadian clock, the control of the circadian clock must first be examined. It is now recognized that mammalian circadian rhythms are controlled by a master circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, as well as by peripheral oscillators located in most tissues (Buijs and Kalsbeek 2001). These circadian clocks are entrained by periodic environmental cues, with the central clock most potently affected by photic stimulation of the retinohypothalamic tract and peripheral clocks synchronized by neurohumoral signals under either direct or indirect control of the central clock (Foster 1998; Balsalobre *et al.* 2000; McNamara *et al.* 2001).

At the molecular level, both central and peripheral clocks mark time with an autoregulatory feedback loop consisting of positive and negative limbs (Reppert and Weaver 2001) (Fig. 1.2). The positive limb of this feedback loop includes at least four basic helix-loop-helix (bHLH) proteins containing PER-ARNT-SIM (PAS) domains, namely two α -class proteins, CLOCK and MOP4, and two β -class proteins, BMAL1 and BMAL2. Heterodimers formed between these α - and β -class proteins bind E-box enhancers (CACGTG) to activate the transcription of genes encoding the negative limb of this autoregulatory feedback loop, as well as genes encoding outputs from the clock. Components of the negative limb are encoded by the *Period* genes (*mPer1*, *mPer2*, and possibly *mPer3*) and the *Cryptochrome* genes (*Cry1* and *Cry2*).

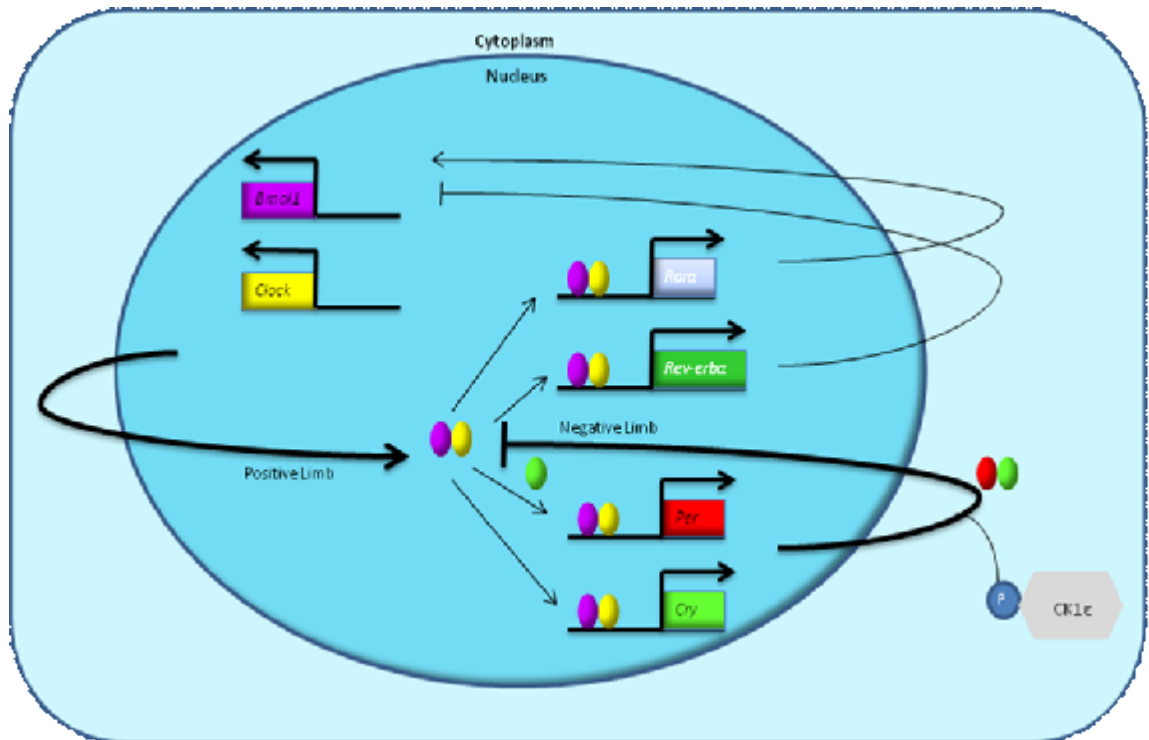


Figure 1.2 – A Model of the circadian clock mechanism

CLOCK (yellow) and BMAL1 (purple) proteins drive the expression of *Per*, *Cry*, *Rev-erba* and *Rora* genes in the nucleus. After dimerisation of PER (red) and CRY (light green) proteins and translocation back into the nucleus, CRY inhibits CLOCK/BMAL1 action and thereby down-regulate their own expression and that of *Rev-Erba* (dark green) and *Rora* (light blue). When REV-ERBA protein is absent, *Bmal1* genes are derepressed and activated by RORα to produce new CLOCK/BMAL1 transcription factors that reinitiate a new circadian cycle. CLOCK proteins are post-translationally modified; CK1ε, for example, phosphorylates mPER2. Adapted from Albrecht and Eichele, 2003.

Following translation of PER and CRY proteins, PER:CRY heterodimers translocate to the nucleus, where they inhibit gene expression driven by α : β heterodimers from the positive limb by interfering with the activation by CLOCK:BMAL1/BMAL2 heterodimers (Kume *et al.* 1999; Griffin *et al.* 1999).

Two additional negative loops of the mammalian circadian clock involve the bHLH transcription factor Differentially Expressed in Chondrocytes (DEC, of which two isoforms have been identified, DEC1 and DEC2) and the nuclear receptor reverse strand c-ERB α (REV-ERB α) (Honma *et al.* 2002; Preitner *et al.* 2002). Both the *dec1/2* and *rev-erba* genes are under direct transcriptional control by the α : β heterodimers. After translation of the corresponding proteins and translocation into the nucleus, both DEC1/2 and REV-ERB α attenuate α : β heterodimers-mediated transcription; DEC1/2 appear to associate with the α : β heterodimers, impairing transcriptional capacity, whereas REV-ERB α specifically represses *Bmal1* transcription, potentially through recruitment of the N-CoR/histone deacetylase 3 co-repressor (Sato *et al.* 2004; Yin and Lazar 2005). Retinoid-related Orphan Receptor (ROR) in the nucleus competes with REV-ERB α to bind to the orphan nuclear receptor target sequence termed RORE sequence (*AAAGTAGGTCA*), in the *Bmal1* promoter. Their opposing effects produce a rhythmic level of BMAL1 and hence CLOCK:BMAL1.

1.6.2.1 – Circadian variations in PAI-1

Laboratory studies have directly linked the body's endogenous circadian clock to PAI-1 rhythmicity. The CLOCK:BMAL1 heterodimer appears to regulate mouse PAI-1, where

Oishi *et al.* (2007) reported the deletion of a putative E-box (located -174 to -179) in the mouse PAI-1 gene resulted in complete inhibition of CLOCK:BMAL1 transactivation of the mouse PAI-1 promoter activity in mouse NIH3T3 cells. Maemura *et al.* (2000) and Schoenhard *et al.* (2003), as well as our group (Chong *et al.* 2006), have recently demonstrated that clock genes can modulate the human *Pai-1* promoter *in vitro*. It was reported that the transfection of either BMAL1 and CLOCK, or BMAL2 and CLOCK expression plasmids up-regulated *Pai-1* promoter activity, with the CLOCK:BMAL2 heterodimer the more potent activator (Schoenhard *et al.* 2003; Chong *et al.* 2006). Furthermore, mutation of either of the two E-boxes in the *Pai-1* promoter fragments drastically attenuated CLOCK:BMAL2 responsiveness, where mutation of the second E-box exerted greater inhibitory effect (Chong *et al.* 2006). The CLOCK:BMAL2 heterodimer transactivates the *Pai-1* promoter by directly binding to the E-boxes, demonstrated with gel shift assays using *in vitro* translated CLOCK and BMAL2, and a 32 bp double-stranded oligonucleotide (5'-CTGGACACGTGGGGAGACAATCACGTG G CTGG-3') probe containing the two E-boxes (Maemura *et al.* 2000).

1.6.2.2 – Non circadian factors affecting oscillations in PAI-1

A number of other factors had been shown to affect the circadian expression of PAI-1 in mice. These include diet (Minami *et al.* 2002; Kudo *et al.* 2004a), the rennin-angiotensin system (RAS; Naito *et al.* 2003; Tsujino *et al.* 2005) and hormones regulating diabetes and obesity (Oishi *et al.* 2004; 2006).

In humans, angiotensin converting enzyme (ACE) inhibition has been reported to alter circadian expression of circulating PAI-1 levels. Brown *et al.* (1998) measured PAI-1 antigen and activity in nine normotensive subjects treated with either a high or low salt diet. Brown and colleagues demonstrated activation of RAS through low salt intake increased PAI-1 antigen during the morning period (8 AM through 2 PM) compared with high salt intake. Furthermore, the interruption of the RAS with the ACE inhibitor quinapril significantly attenuated this diurnal variation in PAI-1 antigen and activity.

Sleep disruption has also been implicated to influence PAI-1 oscillations in humans. Irokawa's group (1998) measured PAI-1 activity in 10 healthy male subjects for two consecutive days. On day one, subjects remained awake all day and night, and slept during the daytime on day two (8:30 AM to 5:30 PM). The overnight wakefulness appear to have significantly blunted the morning peak PAI-1 activity, suggesting that the subjects activities during the night, awake and sleep, substantially affect 24 h oscillations of circulating PAI-1.

Recently, Schoenhard *et al.* (2007), examined whether the entrainment status of the central circadian clock influences PAI-1 rhythmicity. The author detected plasma PAI-1 levels in four blind subjects, two of who were entrained with melatonin and the other two were free-running. It was observed that PAI-1's oscillations were most robust in the two entrained subjects, with peak concentration at 8:17 am. Surprisingly, significant circadian variation in PAI-1 was also observed in the two free-running subjects, with peak concentration at 11:20 am. These results indicate that circadian variation in PAI-1 is independent of both light perception and the body's central circadian clock.

1.6.3 – Regulatory elements in *Pai-1* promoter

The transcriptional regulation of the *Pai-1* gene is complex, and is reflected by the numerous transcription factor regulatory sites characterised so far (Fig. 1.4). van Zonneveld and colleagues (1988) identified a perfect *TATA* box at positions -28 to -23 through sequence analysis, and a glucocorticoid response element (GRE) between the regions -305 and +75 by promoter deletion mapping experiments and studies involving the fusion of *Pai-1* promoter fragments to chloramphenicol acetyltransferase (CAT) gene. The author concluded that a sequence at positions -64 to -59 (5' -*GGAACA*- 3') resembles one half of a GRE (consensus sequence 5'-*GGT(A/T)CA(A/C)NNTGT(C/T)CT*-3'), maybe responsible for glucocorticoid induction. This was later not only confirmed by Brown *et al.* (2000) as mutations in this region abolished the effect of steroid on PAI-1 expression, but demonstrated that the same element was also involved in mediating aldosterone responsiveness. Utilizing techniques such as Methylation interference and DNase I footprinting, Eriksson *et al.* (1998b) demonstrated a very-low-density lipoprotein response element (VLDLRE) within the PAI-1 gene promoter located at -672 to -657. Analysis of the VLDLRE (5'-*TCAGCCGTGTATC*-3') by the author show some homology to the peroxisome proliferator activator response element (consensus sequence 5'-*(A/T)(C/G)A(C/A)CT(A/T)T(G/T)NCC(C/T)* -3'), and this transcription factor may be involved in mediating the induction of *Pai-1* by VLDL. Chen and colleagues (1998) reported two adjacent Sp1 sites (consensus sequence 5'-*(G/T)GGGCGG(G/A)(G/A)(C/T)*-3') located at -73 and -42 that mediates the glucose response in the *Pai-1* gene, as mutations in either of the Sp1 sites resulted in a 50%

decrease of glucose responsiveness in transient transfections of rat aortic muscle cells. Furthermore mutations in both Sp1 sites abolished glucose responsiveness of the *Pai-1* luciferase reporter constructs. To confirm that transcription factor Sp1 was responsible for PAI-1 response to glucose, the author carried out electromobility shift assays (EMSA) and demonstrated electrophoretic mobility shifts of the oligonucleotides comprised of the Sp1 regions was altered by increased glucose concentrations. A hypoxia-response element (HRE) was characterised by Fink *et al.*, (2002) where reporter gene assays demonstrated this HRE (sequence 5'-CACATG-3') located at positions -194 to -187 upstream of the transcriptional start site was necessary, as well as sufficient, for hypoxia-mediated activation of the PAI-1 promoter. Further analysis by EMSA revealed that a hypoxia-inducible protein complex containing HIF-1 α and ARNT interacted with the HRE element. Smith *et al.* (2004) later identified a putative estrogen response element (ERE) located at position -427 in the PAI-1 proximal promoter. This element was examined by site-directed mutagenesis which altered the 3' half-site of the ERE (from 5'-ACTCCACAGTGACC-3' to 5'-ACTCCACAAAGCTT-3'). Transfections of either wild-type or the mutant promoter in bovine aortic endothelial cells (BAECs) demonstrated that the half-site mutation was sufficient to abolish the estrogen receptor alpha (ER α) dependent stimulation of *Pai-1* luciferase activity. The transcriptional responsive elements so far described focus on the proximal *Pai-1* promoter, Hou *et al.* (2004) demonstrated a 5' distal tumor necrosis factor-alpha (TNF α)-responsive enhancer of the *Pai-1* gene located 15kb upstream of the transcriptional start site. This TNF α -responsive element was identified using a combination of DNase I-hypersensitive site analysis and bioinformatics software such as MatInspector and rVISTA. Furthermore the author also shows that the TNF α -

responsive element contained a conserved NFκB site (5'-TGGAATTTCT-3'). EMSA using ³²P-labelled oligonucleotide probe containing the NFκB site resulted in a single new specific band, subsequent studies demonstrated the binding of NFκB subunits p50 and p65 to this enhancer element.

1.6.3.1 – Putative TGF-β1 regulatory elements in the *Pai-1* promoter

The regulation of the human *Pai-1* promoter by Transforming growth factor-beta (TGF-β) has been studied extensively (Fig. 1.5). Keeton *et al.* (1991) incorporated an 800-base pair fragment from the *Pai-1* promoter into a luciferase reporter construct and transfected into Hep3B human hepatoma cells. Initial truncation approaches identified two sequences that were important in mediating the response to TGF-β. The first sequence was located in the proximal promoter (-49 to -87), while the second in a more distal region (-636 to -740). Datta and colleagues (2000) discovered that the proximal sequence of the *Pai-1* promoter contains two Sp1 binding sites, and was required for TGF-β-induced transcriptional activation, since an engineered four-base-pair mutation (GGGTGGGG to GAATTCGG and CCTGCCC to CTAGATC) in both Sp1 sites abolished *Pai-1* promoter activity in response to TGF-β. These same Sp1 sites also mediated glucose response as discussed earlier in this section, and therefore appear to have more than one physiological role in regulating *Pai-1* transcription. The distal region of *Pai-1* promoter was further characterized by Song *et al.* (1998), who found that a 12-base pair sequence from -721 to -732 (AGACAAGGTTGT) was capable of conferring TGF-β responsiveness to a minimal promoter construct. The authors also reported, using EMSA, that SMAD3 and SMAD4 was associated with this element, and

that SMAD4 specifically binds to this region of the *Pai-1* promoter. Dennler and colleagues (1998) further reported that three copies of sequence *AG(C/A)CAGACA* located -730, -580 and -280 in the human *Pai-1* promoter mediate TGF- β transcriptional induction. As mutation of these three sequences (*AG(C/A)CAGACA* to *AG(C/A)TACATA*) was found to attenuate *Pai-1* promoter activity in response to TGF- β stimulation in HepG2, Mv1Lu and NIH 3T3 cells. However, these sequences in the human *Pai-1* promoter were not sufficient to explain the specificity of TGF- β -induced transcription. Another prominent TGF- β response element is the hexanucleotide E-box (5'-*CACGTG*-3'), where Hua *et al.* (1999) reported two copies of this E-box sequence in a *Pai-1* promoter segment (-532 to -794) which was sufficient to confer *Pai-1* promoter response to TGF- β in human HT1080 cells. It was also demonstrated that mutation of the first E-box located at -675 did not diminish promoter activity, but mutation of the second E-box in the region of -559 dramatically reduced promoter activity, hence the second E-box was essential for the activity of *Pai-1* promoter in conferring TGF- β induced transcription. The author then further reported three *AGAC* Smad binding element (SBE) in between the two E-box sequence. Mutation of either the first (-581) or the third (-566) in luciferase reporter constructs (*AGAC* to *CTTG*) markedly reduced TGF- β -induced transcription, in contrast mutation of the second SBE (-577) slightly enhanced TGF- β -induced transcription. These results were confirmed in gel shift assays, where mutation of the first and third SBE dramatically diminished its binding to GST-Smad3, thus demonstrating the first and third SBEs were essential for TGF- β -induced transcription. These data was later supported by Grinberg *et al.* (2003). Interestingly, Hua and colleagues also deleted or added 3, 2 or 1 bp to a 3-bp spacer between the third SBE and second E-box, and found that perturbation in the spacer

impaired TGF- β -induced transcription. It therefore appears that the combination of the E-box, multiple SBE, and a requirement for a fixed spacer between the two binding sites increases the specificity of TGF- β -induced transcription of the *Pai-1* gene.

The transcription factors identified so far that recognize the second E-box and play a role in TGF- β -induced *Pai-1* expression are the basic helix-loop-helix/leucine zipper (bHLHZIP) transcription factors of MYC family including; MYC, MAX, TFE3, upstream stimulating factor (USF1 and USF2). Grinberg *et al.* (2003) reported opposite effects if TFE3 and Max on SMAD3 activation of the *Pai-1* promoter showing that interactions involving the same contact interface can both stimulate and inhibit transcriptional activity. It thus appears that the E-box element likely function as a platform for recruitment of both positive and negative regulators of *Pai-1* expression. UV cross-linking and tethered DNA affinity chromatographic analyses identified USF-1 as a major *Pai-1* E-box recognition factor (Providence *et al.* 2002). Specific E-box mutations that ablate USF-1 binding to a PAI-1 target deoxyoligonucleotide probe (*CACGTG* to ***TCCGTG***) effectively attenuated TGF- β 1-stimulated, *Pai-1* promoter-driven CAT reporter activity (White *et al.* 2000).

Since SMAD proteins interact with many diverse transcription regulatory proteins, and as mentioned earlier the CLOCK:BMAL2 heterodimer act on the E-box in the *Pai-1* promoter, it is plausible that SMAD may interact with the CLOCK:BMAL2 heterodimer at the SBE and the E-box sites, and create a circadian-dependent PAI-1 response to TGF- β family cytokines.

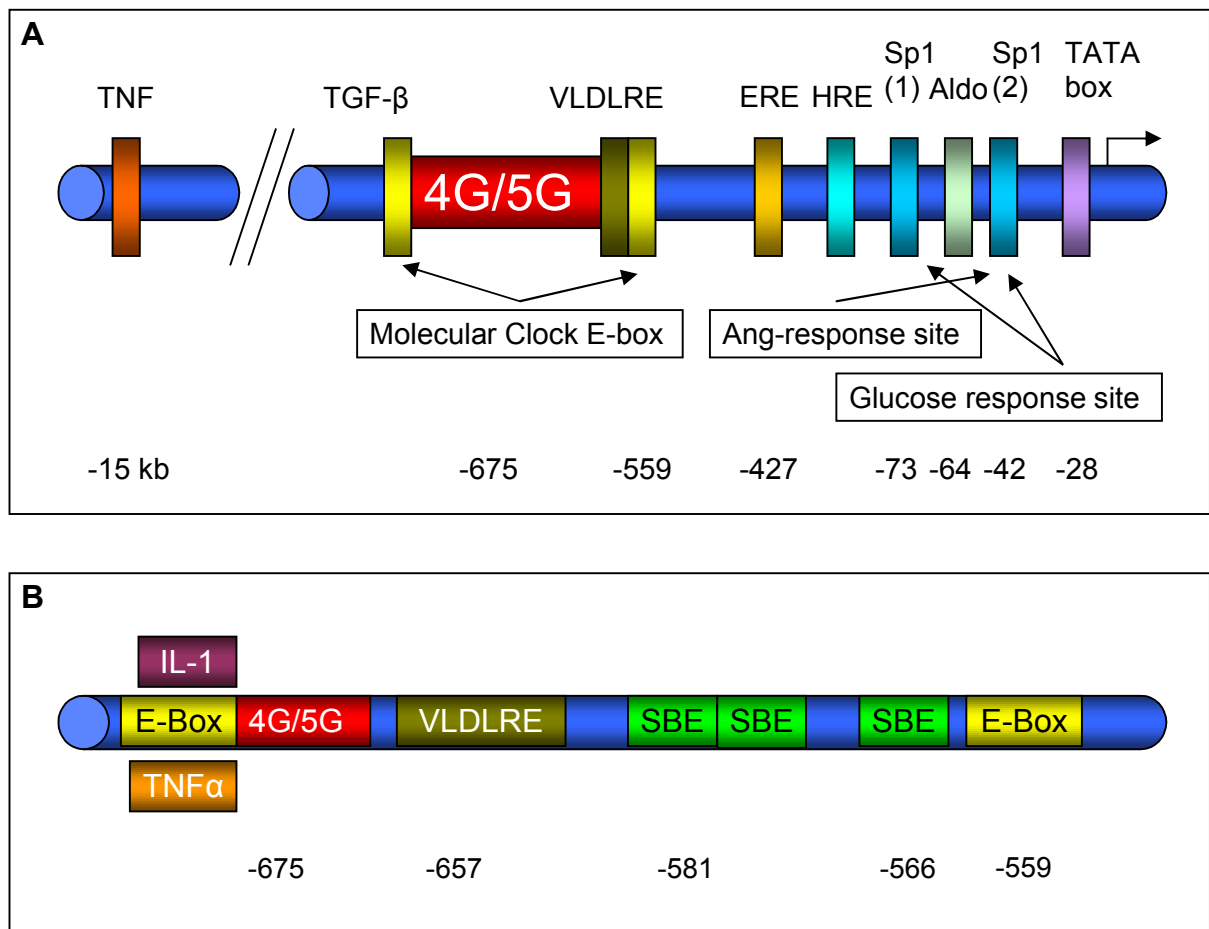


Figure 1.3 – Schematic diagram of the promoter region of the human PAI-1 gene

Some of the important regulatory elements that has been documented and depicted (A) in the schematic diagram include a glucocorticoid response element (GRE) that also mediates aldosterone responsiveness (Aldo), a very-low-density lipoprotein response element (VLDLRE) adjacent to the 4G/5G polymorphism, and two Sp1 sites that appear to mediate glucose and glucosamine responsiveness, tumour necrosis factor (TNF)-responsive element at location approximately 15 kb upstream of the transcription start site, Hypoxia responsive element (HRE), and a putative estrogen response element (ERE). Focusing on the putative elements that are responsive to TGF-β and regions that are adjacent to the 4G/5G polymorphism (B). The human PAI-1 promoter contains two conical E-box (CACGTG), the first is adjacent to the 4G/5G polymorphism site, and the second is situated near Smad binding elements (SBE). It has been reported that TGF-β induced PAI-1 transcription is mediated through the second E-box, and two of the three SBEs. 4G/5G polymorphism dependent PAI-1 transcription has been found for very-low-density lipoprotein (VLDL) and interleukin-1 (IL-1), and CLOCK:BMAL2 heterodimer stimulation. Recently a putative tumour necrosis factor (TNF)-α response element has been reported to be located adjacent to the 4G/5G polymorphism site. The approximate location of the putative elements described is situated on the bottom of the diagram.

1.6.3.2 – Regulatory elements adjacent to the 4G/5G polymorphism

Several transcriptional elements in the *Pai-1* promoter have been reported to be influenced by the 4G/5G polymorphism (Fig. 1.3). The location of VLDLRE is upstream of the 4G/5G polymorphism and may influence VLDL driven *Pai-1* promoter activity. Indeed Eriksson *et al.* (1998b) reported in transient transfections of *Pai-1* promoter fragments in human umbilical vein endothelial cells (HUVEC), that the 4G allele showed a higher promoter activity in response to VLDL than the 5G allele. They also went on to show with EMSA that the 5G allele-specific repressor can compete for binding to the *Pai-1* promoter with both the common transcriptional activator and the VLDL-induced factor. Swiatkowska *et al.* (2005) demonstrated that TNF α induced expression and gene transcription of *Pai-1* involves the regulatory region between -664 and -680. This was demonstrated in EMSA experiments containing oligonucleotide of the putative κ B-binding site in the PAI-1 promoter binding to transcriptional factor subunits p50 and p65, which could be abolished with mutation of oligonucleotide.

Additionally, mutation of the putative regulatory element attenuated *Pai-1* promoter responsiveness to TNF α . Since the putative regulatory element is situated next to the 4G/5G polymorphism, the author hypothesised that the polymorphism may also influence *Pai-1* promoter activity to TNF- α . However, additional experiments were not carried out to confirm this hypothesis. The induction of *Pai-1* gene by IL-1 also appears to be influenced by the polymorphism, since IL-1 induced *Pai-1* promoter activity in the *Pai-1* construct containing the 4G allele, but not in the 5G carrying construct (Dawson *et al.* 1993). We have also reported that the 4G/5G polymorphism is a critical element in

regulating *Pai-1* circadian expression (Chong *et al.* 2006). It was observed that the *4G* polymorphism enhanced the expression of *Pai-1*; luciferase promoter constructs more than the *5G*, when activated by CLOCK/BMAL2 heterodimer. Since these experiments were carried out using COS7 cells, it is not known whether this hypothesis would be physiological relevant in other cell types such as monocytes and platelets.

1.7 – Hypothesis

Previous data has shown CLOCK:BMAL2 heterodimer is capable of inducing PAI-1 gene expression in endothelial cells and simian kidney cells. We propose that clock genes also regulate PAI-1 expression in monocytes and platelets, and furthermore the interaction of clock genes with the *4G/5G* region of the PAI-1 promoter is a key regulator of PAI-1 synthesis in these cell types. This would contribute to variability demonstrated in the resistance to fibrinolysis and thrombolysis.

1.8 – Aim

The aim of the thesis was to determine whether the production of PAI-1 by blood cells that are physiological relevant in haemostasis and thrombosis, is regulated through the *4G/5G* promoter polymorphism and/or the peripheral clock. This aim is further divided into different areas of investigations as described below:

- to investigate if the interaction of platelets with monocytes resulted in monocyte PAI-1 expression, and to evaluate the role of direct cell to cell

contact compared to soluble mediators in provoking this response, and explore whether this is influenced by the peripheral circadian clock and/or the *4G/5G* polymorphism.

- to examine whether a peripheral circadian clock exists in monocytes, and how it may be affected during inflammatory or thrombotic stimulus.
- to assess if platelets can synthesise PAI-1, and whether this synthesise or release of PAI-1 following activation is dependent on the *4G/5G* polymorphism.
- to evaluate the influence of the circadian clock and *4G/5G* polymorphism on thrombolysis in two model systems.

Chapter 2

Materials and methods

2.1 – Monocyte isolation

Peripheral blood was collected from 10 healthy volunteers with their informed consent, and was used throughout this study. Blood was drawn into a 4.5 ml volume of 3.2% wt/vol sodium citrate tube (BD Vacutainer, Oxford, United Kingdom). Aliquots of 1 ml of the blood were incubated at 37°C for 0, 1.5, 4, 6, and 24 h, either without stimulant, or with 200 ng/ml Lipopolysaccharide (LPS, *Escherichia coli* 0111B:4, Sigma-Aldrich, Dorset, UK), or 500 ng/ml cross-linked collagen-related peptide (XL-CRP from Dr Richard Farndale, University of Cambridge).

At each time point, monocytes were isolated by positive selection on CD14 magnetic beads (Dynal, Wirral, United Kingdom) and mRNA was extracted using Oligo(dT)₂₅ beads (Dynal). The 1ml blood samples were transferred to tubes containing 100 µl CD14 beads (4×10^7 beads) and mixed thoroughly by pipetting. Next the blood was incubated for 10 min at 4°C with rotation. The tube containing the blood was then placed on a Dynal Magnetic Particle Concentrator (MPC, Dynal) for 4 min to separate the CD14 beads. The blood was removed, and the CD14 beads were washed twice with 500 µl cold phosphate buffered saline (PBS, Sigma-Aldrich). After washing, 1 ml of lysis/binding buffer (100 mM tris-HCL, pH 7.5, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM dithiothreitol) was added to the CD14 beads with monocytes attached, and placed in the magnet for 5 min at room temperature to allow lysis to occur. The lysate was then transferred to a new, sterile vial and the extraction of mRNA proceeded as described in section 2.5.

2.2 – Analysis of monocyte-platelet aggregates

Blood was drawn into citrated tubes, and placed into microtubes, followed by activation with 500 ng/ml (final concentration) of CRP-XL. Unstimulated controls were incubated in parallel. The P-selectin blocking antibody 9E1 (R&D systems, Abingdon UK) were added at 25 µg/ml final concentration. Blood was incubated at 37°C for 1.5 h and 4 h. At each time point, 5 µl aliquots were removed and added to HBS (10mM HEPES; 150mM NaCl; 1 mM MgSO₄; 5 mM KCl; pH 7.4) containing mouse IgG1 to block non-specific fc bridge (MOPC31C, Sigma-Aldrich), an R-phycoerythrin (RPE)-Cy-5-conjugated antibody against monocyte marker CD14 (Dakopatts AB, Stockholm, Sweden), and RPE-CD42b (Dakopatts AB). After 30 min incubation, the reaction was stopped with 500 µl 0.2% formaldehyde saline and the samples analysed in the flow cytometer. The flow cytometer was triggered by forward and side scatter and the fluorescent signal (RPE-Cy5) of the monocyte marker (CD14) were analysed in a 2 colour histogram. To prevent analysis of irrelevant signals, a discriminator on FL4 events was set at 6 arbitrary units. Marker-positive events were allocated to monocyte on the basis of forward and side scatter. Monocyte-platelet aggregates were identified as those events positive for both the monocyte marker CD14 and the platelets marker CD42b-RPE, whilst free monocytes were events positive only for the monocyte marker.

2.3 – Studies of Platelet-Monocyte Interaction

Human mononuclear cells (MNCs) were isolated using Lymphoprep (Axis-Shield, Oslo, Norway). Blood was drawn into a 4.5 ml volume of 3.2% wt/vol sodium citrate tube. Whole blood (2.5 ml) was mixed with 2.5 ml of PBS. The mixture was then underlayered with 3.5 ml Lymphoprep. After centrifugation of 30 min at 3000 rpm, the interface layer was removed and transferred into another centrifuge tube. Equal amounts of PBS were mixed with the plasma, and then centrifuged for 15 min at 1600 rpm. The supernatant was then removed, to leave an isolated mononuclear cell preparation.

Platelets were isolated by centrifugation of whole blood for 20 min at 900 rpm, to produce platelet-rich plasma (PRP). Platelet releasates were prepared by exposing 1 ml PRP to CRP-XL for 10 min at 37°C. The platelet-rich plasma was then centrifuged for 15 min at 3000 rpm, and aliquots (500µl) of purified platelet releasates were added to the isolated mononuclear cells in the presence or absence of blocking antibodies to 100 µg/ml TGF-β1 (R&D systems, Minneapolis, USA), 1 µg/ml IL-1(R&D systems), or both. Stimulation of MNCs by rP-selectin (20 µg/ml; R&D systems), and rTGF-β1 (0.4 ng/ml; R&D systems), was also carried out. Samples left unstimulated were incubated in plasma for 4 h at 37°C. After the incubation, monocytes were isolated with CD14 magnetic beads as described in section 2.1. The extraction of mRNA and the process of reverse-transcription were followed as described in sections 2.5 and 2.7.

2.4 – Platelet Isolation and plasma collection

Blood was collected into a 4.5 ml volume CTAD tubes (BD Vacutainer), containing buffered sodium citrate (3.2%), theophylline, adenosine, dipyridamole (0.3 mL). The CTAD tubes were centrifuged at either 900 rpm for 20 min to produce PRP, or 10,000 rpm for 15 min to collect platelet poor plasma (PPP). Platelet and plasma was isolated at two different time points; for morning samples it was approximately 9 am (± 20 mins) and for afternoon samples at 4 pm (± 20 mins). At each of the two time points, plasma were collected into 1.5 ml microtubes after centrifugation and PAI-1 antigen was measured by ELISA as described in section 2.11. The isolation of platelets was carried out by negative selection using pan Mouse IgG beads (Dynal) in combination with a CD11a mAb antibody (to remove lymphoid and myeloid cells), and CD14 magentic beads (to deplete any residual monocytes).

To coat the IgG beads with CD11a mAb antibody, 100 μ l of IgG beads and 1 ml PBS were pipetted into a vial and mixed. The vial was placed on the MPC and the supernatant was discarded. The IgG beads was then resuspended in 100 μ l of PBS, followed by the addition of 10 μ l CD11a mAb. The mixture was incubated for 30 min at room temperature on a rotary mixer. The mixture was placed on the MPC and the supernatant was discarded. The IgG beads coated with CD11a mAb antibody was resuspended with 100 μ l of PBS.

To negatively isolate platelets, 1 ml of PRP was transferred into a sterile vial with 50 μ l of pre-coated IgG beads with CD11a mAb antibody. After incubating at room

Materials and methods

temperature for 20 min, the vial was placed on the MPC and supernatant was transferred into a new clean vial with the addition of 100 μ l CD14 beads. The vial was left to incubate 15 min at room temperature on a rotary mixer, and then placed on the MPC and supernatant was transferred into a new clean vial. The vial was then centrifuged for 20 min at 3,000 rpm to pellet platelets, with the supernatant discarded afterwards. 1 ml lysis/binding buffer was then pipetted onto the platelet pellet, and incubated at room temperature for 5 min. The extraction of mRNA is described in section 2.5. For platelet stimulation, the platelet pellet was resuspended with HGC buffer (HBS plus 5 mM glucose, CaCl_2 2 mM) and adjusted to a count of $200 \times 10^3/\mu\text{l}$. Hirudin 10 U/ml (Sigma-Aldrich) was added to the platelet suspension.

Aliquots (1 ml) of platelet suspension were exposed to the final concentration of either CRP-XL 100 $\mu\text{g/ml}$, TRAP $1 \times 10^{-3}\text{M}$ (Bachem, Weil am Rhein, Germany) or ADP $1 \times 10^{-3}\text{M}$ (Sigma-Aldrich), with the TRAP stimulation in the presence and absence of Cycloheximide 100 $\mu\text{g/ml}$ (Sigma-Aldrich), for 10 min, 1 h, 2 h, 4 h and 8 h. After incubation, the PRP was centrifuged for 15 min at 3000 rpm, and the platelet poor plasma was removed and analysed for PAI-1 antigen using an ELISA as described in section 2.11. Additionally 1 ml lysis/binding buffer was pipetted onto the platelet pellet, and incubated at room temperature for 5 min to examine *Pai-1* mRNA transcript. The extraction of mRNA was carried out as described in section 2.5.

2.5 – mRNA extraction

For extraction of both monocyte and platelet mRNA, 100 µl of washed Oligo(dT)₂₅ beads were added to monocyte or platelet lysates. This mixture was incubated for 5 min, with rotation, at room temperature. The vial was then placed on the magnet and the supernatant removed. The Oligo(dT)₂₅ beads were washed twice with 250 µl washing buffer A (100 mM tris-HCL, pH 7.5, 500 mM LiCl, 10 mM EDTA, 1% LiDS), then twice with 250µl washing buffer B (100 mM tris-HCL, pH 7.5, 500 mM LiCl, 10 mM EDTA). The Oligo(dT)₂₅ beads were then resuspended in 20 µl of water, and reverse transcriptase PCR was performed immediately, as described in section 2.7.

2.6 – Extraction of RNA from whole blood

RNA was isolated from whole blood at two different time points; for morning samples it was approximately 9 am (±20 min) and for afternoon samples at about 9 pm (±20 min). Blood from 7 donors was collected into 2.5 ml volume PAXgene blood RNA tube (QIAGEN, USA) and incubated at room temperature for 2 h. Purification of the RNA began with a centrifugation step of 9,300 rpm for 10 min to pellet nucleic acids in the PAXgene blood RNA tube. The pellet was washed with 4 ml RNase-free water and resuspended with 350 µl resuspension buffer, 300 µl binding buffer and 40 µl proteinase K for protein digestion. The mixture was incubated for 10 min at 55°C in a shaker-incubator at 1000 rpm. After incubation, the lysate was centrifuged through a Shredder spin column for 3 min at 10,000 rpm, which homogenized the cell lysate and removed residual cell debris. The supernatant of the flow fraction was transferred to another 1.5

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ml tube. 350 µl ethanol was added to adjust binding conditions and pipetted into an RNA spin column. The column was centrifuged for 1 min at 9,300 rpm to allow the RNA to bind on the silica membrane, and contaminants to pass through. The remaining contaminants were removed with the addition of 500 µl wash buffer followed by a centrifugation step of 9,300 rpm for 1 min. This washing step was repeated four times. Between the first and second wash steps, 10 µl DNase I stock solution and 70 µl DNA digestion buffer was added to the membrane, and incubated for 15 mins at room temperature to removed bound DNA. After the wash steps, the RNA was eluted from the column with 40 µl of elution buffer and a centrifugation step of 9,300 rpm for 1 min. The RNA yielded was then used as template for Reverse transcriptase PCR, which was preformed as described in section 2.7.

2.7 – Reverse transcription PCR

First-strand cDNA preparations were obtained using SuperScript II Reverse Transcriptase (Invitrogen, Paisley, UK). Firstly a mix was prepared containing 1µl mRNA (approx. 2 ng), 10 mM Oligo(dT) (Invitrogen), 10 mM dNTP (Eppendorf, Hamburg, Germany), and double distilled water to make a final volume of 20 µl. The reaction was heated to 65°C for 5 min and then chilled on ice with 4 µl 5X first strand buffer (Invitrogen, 250 mM Tris pH 8.3, 375 mM KCl, 15 mM MgCl₂), and 2µl dithiothreitol DTT (Invitrogen, 0.1 M) added. After gentle mixing, 1 µl Superscript II reverse transcriptase (Invitrogen, 200 U/µl) was added. The mixture was then incubated at 42°C for 50 min, and finally heated to 70°C for 15 min.

2.8 – Semi-quantitative PCR

From these templates, cDNA encoding hPAI-1 and various human clock genes was amplified using the conditions given below. A master mix was made containing: 10 ng template DNA, 0.8 mM dNTP mix, 0.5 μ M of each primer (Invitrogen), 10 μ l Reaction buffer (ABgene, Epsom, UK), 75 mM Tris-HCl pH 8.8, 20 mM $(\text{NH}_4)_2 \text{SO}_4$, 0.01% v/v Tween 20), 1.5 mM MgCl_2 (ABgene), 0.05 U/ μ l Taq polymerase (ABgene), water to a final volume of 20 μ l.

Each cycle consisted of:

- 94°C for 1 min
- 59°C for 1 min
- 72°C for 2 min

The number of cycles used for each gene and with the different samples is discussed in the results section 3.2.1.1, and details of each set of primers are given in table 1.1. The PCR products were loaded on to a 2% agarose gel (MP Biomedicals, London, UK) made with 1x Tris-Acetate-EDTA (TAE, 40 mM Tris/Acetate, 1 mM Na_2EDTA), and then subjected to electrophoresis at 80V for 45 min, after which the image of each band was recorded using the Genesnap imaging system and programme (Syngene, Cambridge, UK). The intensity of the PCR products on the gel was determined using the Genetool programme (Syngene). The mRNA level for each target gene was calculated relative to the 18S housekeeping gene, and then standardized to the 0 h samples.

Gene	Region amplified	Primers (5' to 3')	GC Content (%)	Melting Tempertaure (°C)
<i>18s</i>	+1349 to +1548	<i>GTGGAGCCATTTGTTTGGTT</i> ^a <i>CGCTGAGCCAGTCAGTGTAG</i> ^b	50 60	55.5 57.5
<i>Pai-1 Set 1</i>	+627 to +1081	<i>TGCCCTCTACTTCAACGGCC</i> ^a	60	60.7
<i>Pai-1 Set 2</i>	+657 to +1081	<i>CAACGGCCAGTGGAAGACTC</i> ^a <i>CTTGGTCTGAAAGACTCGTGAAG</i> ^b	60 47.8	58.3 55.4
<i>Dec1</i>	+575 to +1037	<i>GCTGGTGAGCTGTCAGGGA</i> ^a <i>CTTGCTTAATTGCGCCGAT</i> ^b	63.2 47.4	59.7 54
<i>Bmal2</i>	+489 to +801	<i>GAGATCTTTAAAAGGCTTGACA</i> ^a <i>CAAACCAGTTTTGGCATCTATT</i> ^b	36.4 36.4	50.7 51.8
<i>Bmal1</i>	+511 to +853	<i>CCACTGACTACCAAGAAAGCAT</i> ^a <i>CCATCTGCTGCCCTGAGA</i> ^b	45.5 61.1	54.9 57.1
<i>mPer2</i>	+950 to +1246	<i>CCGGAAAAGCCACGAGAATG</i> ^a <i>CTGTCACTAGGGTGGAGCTG</i> ^b	55 60	56.5 57.1
<i>Clock</i>	+1103 to +1421	<i>CATGCTTCCTGGTAATGCTA</i> ^a <i>CTCTGAATGTTCCCCTTCTG</i> ^b	45 50	52.3 52.7

Table1.1 – Primer sequence used to amplify *Pai-1* and various clock genes in semi-quantitative PCR.

The regions amplified with a positive number show downstream sequences with respect to transcription start site. Where ^aSense and ^bAnti-sense strands. *Pai-1* primer sets 1 and 2 share the same anti-sense primer.

2.9 – Quantitative (real time) PCR

Pai-1 mRNA expression was also measured by quantitative real-time PCR to validate the results obtained from semi-quantitative PCR, using a light cycler machine (Applied Biosystems, California, USA). Two types of chemistry were used to detect PCR products.

- TaqMan (also known as fluorogenic 5' nuclease activity),
- SYBR Green I dye.

The TaqMan chemistry uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles. The SYBR green chemistry uses SYBR Green I dye, a highly specific, double-stranded DNA binding dye, to detect PCR products as they accumulate during PCR cycles. The most important difference between the TaqMan and SYBR green I dye chemistries is that SYBR green I dye will detect all double-stranded DNA, including non-specific reaction products, where the Taqman probe are gene-specific.

PCR amplification for the SYBR green method was performed with PCR MasterMix (Applied Biosystems) containing the target primers for *18s* and *Pai-1* details are given in table 1.2), Hot Star *Taq* DNA polymerase, SYBR Green and PCR buffer. Samples using SYBR green chemistry were subjected to an initial denaturation step at 50°C for 2 min then at 95°C for 10 min, followed by 40 PCR cycles each, comprised 95°C for 45 s,

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59°C for 45 s and 72°C for 45 s, finally a dissociation step of 95°C for 15 s, 60°C for 15 s and 95°C for 15 s was added to allow any non-specific products to be detected.

The TaqMan method was performed using a PCR MasterMix (Applied Biosystems) containing the target probes (18S and PAI-1), Hot Start *Taq* DNA polymerase, TaqMan and PCR buffer. The 18S TaqMan probe was derived from GenBank ID X03205 and the TaqMan probe for *Pai-1* was produced from GenBank ID M16006 binding onto exons 2 and 3. All samples were analysed in triplicate. The TaqMan method also involved an initial denaturation step at 50°C for 2 min then 95°C for 10 min, followed by 40 PCR cycles each comprising of 95°C for 15 s, 60°C for 1 min.

As *Pai-1* and *18s* mRNA were amplified, the increase in fluorescence was measured in real time. The threshold cycle, which is defined as the cycle number at which the fluorescence reaches 10 x the standard deviation of the baseline, was calculated and the relative gene expression of *Pai-1* was calculated in comparison to *18s*. A similar set-up was used for the controls except that the reverse transcriptase was omitted and no PCR products were detected under these conditions.

2.10 – Genotyping

To determine the polymorphism in the promoter of the gene for *Pai-1*, blood samples were taken from the same healthy volunteers used for monocyte isolation. Genomic DNA was isolated from blood cells using FlexiGene method (Qiagen, Crawley, United Kingdoms). The process firstly involved mixing 10 ml buffer FG1 (lysis buffer) with 4

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ml of whole blood in a 50 ml centrifuge tube, which was then centrifuged for 5 min at 3000 rpm. The supernatant was discarded, and 2 ml buffer FG2 (denaturation buffer) and protease were added into the tube. The sample was then incubated in a water bath at 65°C for 10 min. After the incubation step, 5 ml isopropanol (100%) was mixed in the tube for DNA precipitation. The mixture was then centrifuged for 3 min at 3000 rpm, the supernatant discarded, and 5 ml ethanol (70%) was added to wash the DNA. Again, the mixture was centrifuged for 3 min at 3000 rpm, and the supernatant discarded. Finally, 1 ml buffer FG3 (hydration buffer) was pipetted into the tube, and the DNA was dissolved for 1h at 65°C in a water bath.

Using the genomic DNA as template, PCR reactions were set up to amplify the region – 534 to –811 in the *Pai-1* promoter using a combination of forward primer (5'-ATACGCGTAAGCTTTTACCATGGTAAC-3') and reverse primer (5'-CCCAACAGCCACAGGGCATGC-3'). The PCR conditions used in these reactions were as described in section 2.8 with 30 cycles. A master mix was made containing: 100 ng template DNA, 0.8mM dNTP mix, 0.5 µM of each primer, 10 µl Reaction buffer (75mM Tris-HCl pH 8.8, 20mM (NH₄)₂ SO₄, 0.01% v/v Tween 20), 1.5 mM MgCl₂, 0.05 U/µl Taq polymerase, water to a final volume of 100 µl.

The resulting amplicons were subjected to gel electrophoresis for verification, and then extracted using QIAquick Gel Extraction Kit (Qiagen). The DNA fragment from the gel was excised, and weighed. The slice of gel was incubated with buffer QG (solubilization and binding buffer, 3 volumes to 1 volume of gel) at 50°C for 10 min. Once the gel slice was completely dissolved, 1 gel volume of isopropanol was added to

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the sample. The DNA was applied to the QIAquick column, and centrifuged for 1 min. The flow-through was discarded, and another 0.5 ml of buffer QG was added to the column, and centrifuged for 1 min. The DNA was washed by adding 0.75 ml of buffer PE (ethanol), and centrifuged for 1 min. The flow-through was discarded, and the column was then centrifuged for an additional 1 min at 13,000 rpm. The DNA was eluted by placing the column in a clean 1.5 ml microcentrifuge tube with the addition of 50 of buffer EB (10 mM Tris-HCl, pH 8.5), and centrifuged for 1 min.

The identities of all resulting purified PCR products were sequenced using the automated sequencing facility at the Protein and Nucleic Acid Chemistry Laboratory (PNACL) located in the University of Leicester.

2.11 – Enzyme-Linked Immunosorbent Assay (ELISA)

To prepare plasma samples, CTAD tubes containing whole blood from healthy donors were centrifuged at 3000 rpm at 20°C for 30 min. The plasma was then transferred into a new 1.5 ml microtube and a second centrifugation was applied at 10,000 rpm for 15 min. The plasma was pipetted into a clean 1.5 ml microtube and stored at -80°C until analysed by enzyme-linked immunosorbent assay (ELISA).

In these experiments, ELISA (Hyphen BioMed, Neuville-sur-Oise, France) is used to detect the level of PAI-1 in a sample of plasma. It utilises two antibodies, which are specific to PAI-1, one on the solid phase and the other coupled to an enzyme (horse radish peroxidase). The ELISA procedure consisted of firstly, restoring PAI-1 standard

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with 2 ml of F-sample diluent (SD) and PAI-1 control I + II in 1ml water. These samples were then diluted with SD; where the PAI-1 control I + II was diluted 1:5, and the PAI-1 standards were diluted to produce a set a control of known concentration from 0 ng/ml to 10 ng/ml. Simulated samples were diluted 1:10 with SD, so that the PAI-1 concentrations were in the optimal range of detection by the ELISA kit.

After preparing the dilutions, 100 µl SD was pipetted into each well, along with either 100 µl of the control, standards or sample on the plate. The mixture was then placed on a plate shaker for 2 min. The plate was left to incubate for 2 h at room temperature followed by 5 successive washings with washing buffer. Next, 200 µl immunoconjugate (IC) added into each well and left to incubate for a further hour, which was again followed by 5 successive washings. 200 µl TMB (peroxidise substrate, 3,3',5,5' - tetramethylbenzidine) was then added to each well and left to incubate for 5 min. To stop the reaction, 50 µl sulphuric acid (0.45 M) was pipetted into each well with a further incubation at room temperature for 10 min. The absorbance was then measured on a plate reader at 450 nm.

2.12 – Preparation of Thrombi *in Vitro*

Two systems were used to produce thrombi in vitro, the Chandler loop and Thromboelastometry (TEM).

2.12.1 – Chandler Loop

The Chandler Loop system (Chandler, 1958) consists of a cylinder that is rotated at an angle 30° to the horizontal at 12 revolutions per minute. The cylinder has a diameter of 10 cm and a length of 5 cm. The cylinder base is 12 cm in diameter as to give a lip of 1 cm around the base onto which the Chandler loops can be mounted.

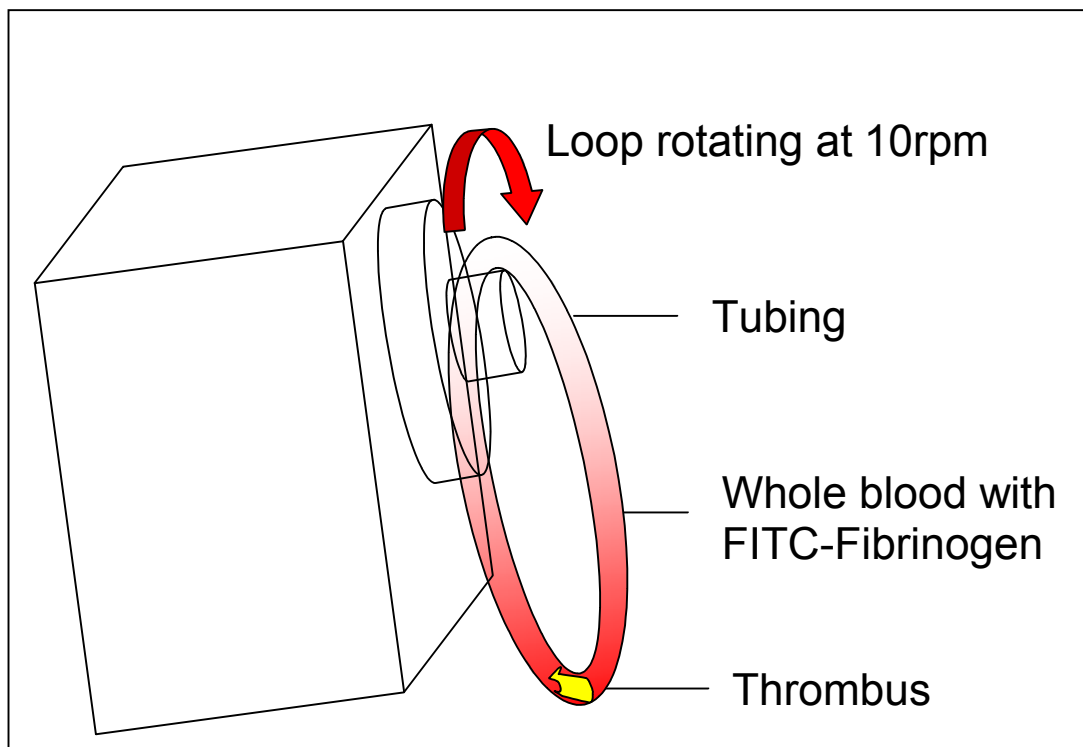


Figure 2.1 – Setup of Chandler loop model

Thrombi formed in Chandler loop mimics human arterial thrombi. This is achieved through the movement of the blood, which is rotated in non-sterile tubing at 10 rpm for 2 h.

The non-sterile tubing (internal diameter of 3.0 mm, external diameter of 4.2 mm; *Protex Limited*) was prepared by cutting into 45 cm in length. Blood was taken into citrated tubes from the same volunteers as used in the previous studies. 900 µl of this citrated blood was then recalcified with 100 µl of 0.25 M CaCl₂, and mixed together with 150 µl 0.9% saline, 50 µl FITC-Fibrinogen (preparation of FITC-Fibrinogen explained later in section 2.12.1.1). This mixture was then gently added to the tubing, to avoid the introduction of air bubbles, the two ends of the tube were joined to form an enclosed loop using a short section of tubing (4.2 mm diameter) to form a cuff. The loop was then mounted onto a cylinder and rotated for 2 h at 10 r.p.m, at room temperature. After this time the clot was removed from the tube and washed with Hanks Buffer, it was then blotted and weighed, and then placed in 1.5 ml of Hanks buffer in a microtube (warmed to 37°C). The clot was then left to incubate for 24 h at 37°C and then weighed. To measure fibrinolysis, 1 ml of supernatant from the microtube was transferred into a cuvette and analysed on the spectrofluorophotometer. The detection of FITC-fibrinogen was carried out using the wavelengths 495 nm (excitation) and 525 nm (emission).

2.12.1.1 – Preparation of FITC-fibrinogen

A column was prepared by pre-swelling Sephadex (Sigma-Aldrich) in water for 24 h at 4°C. It was then layered gently into the column so not to form bubbles, and washed with phosphate buffered saline (PBS, Sigma-Aldrich). Fibrinogen (Sigma-Aldrich) was diluted to 1 mg/ml with 0.1 M sodium carbonate and dialysed for 24 h, at 4°C. FITC (Sigma-Aldrich) was then prepared to a concentration of 10 mg/ml with dimethyl

sulphoxide (DMSO). To conjugate fibrinogen with FITC 1 mg/ml of fibrinogen was added to a bijou, along with 10 ng/ml of FITC solution. The FITC/fibrinogen mixture was then wrapped in aluminium foil and placed on a rotary stirrer for 2 h at room temperature. To separate the bound and free FITC, the FITC/fibrinogen mixture was layered onto the sephadex Gb column with the addition of PBS. The first yellow band was collected (FITC-fibrinogen), leaving behind a brighter yellow band which was unbound FITC. FITC-fibrinogen ratio was measured on spectrophotometer.

2.12.2 – Thromboelastography

Thromboelastography (TEG) is a technique used to assess the haemostatic properties of whole blood. TEG measures the forces within a blood sample as it clots. As clotting initiates, fibrin strands form, binding platelets and blood cells together. Physically, this alters the elastic shear and torque forces of the blood sample. An alternative version is the rotation thromboelastometry (designated as TEM in the present study), in which the sensor shaft rather than the cup which rotates (ROTEM; Pentapharm GmbH, Munich, Germany).

300 µl of citrated whole blood was taken from the same donors as used in previous experiments and mixed with 25 µl 0.25M CaCl₂ for the initiation of clotting, and 5 µl 0.175 µg tPA to enhance fibrinolysis. A pin (sensor), suspended on torsion wire was lowered into the sample. The pin is fixed on the tip of a rotating shaft which is guided by a ball bearing system. The shaft rotates back and forth (+/- 4.75°; cycle time 10/min). It is connected with a spring for the measurement of elasticity. As blood clots, fibrin

strands form between the pin and the clotting blood. The pin is therefore subjected to the changes in elastic shear experienced in the clot. These changes acting on the pin are detected by an optical detection system, which transmit the measurements to a computer for processing. Through TEM, several clot formation parameters were recorded including clotting time (CT), clot formation time (CFT) and maximal clot firmness (MCF). Fibrinolysis as measured in TEM represents the size of thrombi after 60 minutes from when the MCF was detected.

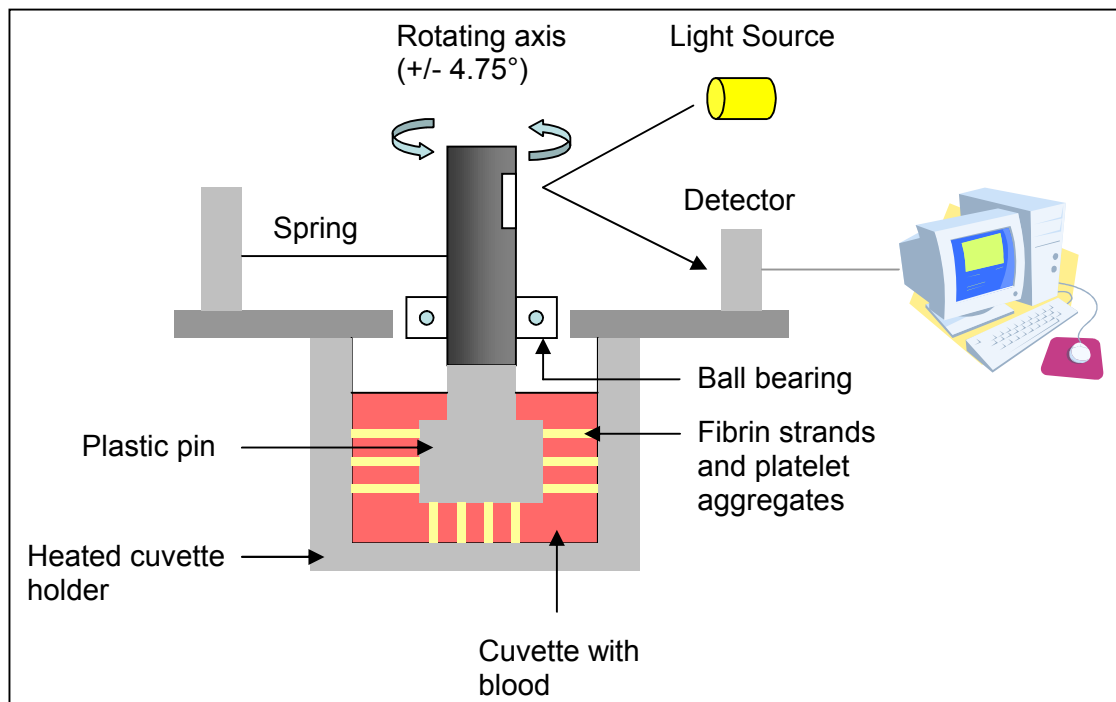


Figure 2.2 – Setup of the TEM system

The rotation of the pin in the cup of blood allows for the detection of both thrombosis and thrombolysis. As the clot forms, the resistance of pin movement increases. However, lysis of the clot results in decreased resistance of pin rotation. The differential resistance on pin rotation is measured through an optical detection system, and ultimately processed by a computer.

2.13 – Statistical analysis

Data in this thesis are expressed as mean \pm SEM. GraphPad prism was employed for all the statistical analysis. ANOVAs were used (one and two-way) to analyse the data, together with appropriate *a posteriori* tests.

Chapter 3

Regulation of monocyte *Pai-1* mRNA and protein expression

3.1 - Introduction

Monocytes are a major cell type in the initiation and propagation of a developing thrombus. During thrombosis, monocytes interact with platelets and thereby become incorporated into a thrombus. Previous studies have demonstrated the production of PAI-1 by monocytes subjected to an inflammatory stimulus (Hamilton *et al.* 1993; Lundgren *et al.* 1994), however, it is unknown whether monocytes are capable of synthesising PAI-1 in conditions associated with thrombosis. This section of the report examines the regulation of monocyte *Pai-I* mRNA and protein expression by activated platelets, and the role of direct cell to cell contact compared to soluble mediators in provoking this response. Furthermore, this section also investigated whether the 4G/5G polymorphism was involved in mediating monocyte *Pai-I* in response to aggregating platelets.

3.2 - Results

3.2.1 - Activated platelets induce PAI-1 expression in monocyte

To determine whether the platelets induce *Pai-I* expression in monocytes, whole blood was taken at 9 am (± 10 min) and incubated with cross-linked collagen related peptide (CRP-XL) to stimulate platelets, controls consisted of monocytes with lipopolysaccharide (LPS) or left untreated at 37°C. *Pai-I* mRNA expression was determined by real-time PCR in extracted monocytes for 1.5-24 h (Fig. 3.1). The concentration of CRP-XL used was 500ng/mL, which has been shown by others in the

lab to maximally stimulate platelets as measured by monocyte platelet aggregate formation. Use of the platelet-specific agonist CRP-XL ensured that monocytes themselves were not directly activated. The mRNA level for *Pai-1* was calculated relative to the *18s* housekeeping gene from the same cDNA. The data was standardized against the 0 h sample and log transformed. Analysis of variance (ANOVA) revealed significant Agonist ($F_{(2,60)} = 27.11$, $P < 0.001$), Time ($F_{(4,60)} = 181.22$, $P < 0.001$) effects, and A x T ($F_{(8,60)} = 8.09$, $P < 0.001$) interactions, indicating that the type of agonist and time points interact in their effect on monocyte *Pai-1* mRNA levels. Furthermore, Bonferroni tests showed that stimulation of monocytes with CRP-XL resulted in a statistically significant rise in the abundance of *Pai-1* mRNA in monocytes compared to samples at baseline, in all time points, increasing 200-fold at 1.5 h ($P < 0.001$) and reaching maximum expression at 4 h (5500-fold, $P < 0.001$), followed by a modest reduction at 6 h (4700-fold, $P < 0.001$), and falling to 1300-fold ($P < 0.001$) by 24 h. In contrast, *Pai-1* expression only reached statistical significant at 24 h (386-fold, $P < 0.01$) in monocytes incubated with LPS. In these tests, the stimulation by CRP-XL or activated platelets clearly provoked a larger response in monocyte *Pai-1* expression as compared with that of LPS. Consistent with *Pai-1* mRNA expression, ANOVA of PAI-1 antigen data (Fig. 3.1) gave highly significant effects for Agonist ($F_{(2,108)} = 38.59$, $P < 0.001$), Time ($F_{(4,108)} = 153.99$, $P < 0.001$) and A x T interactions ($F_{(8,108)} = 39.96$, $P < 0.001$), demonstrating that the type of agonist and time points interact in their effect on monocyte PAI-1 protein levels. Bonferroni tests revealed CRP-XL stimulated whole blood produced significant higher levels of PAI-1 antigen in comparison to monocytes in resting conditions. Induction of PAI-1 antigen was seen at 1.5 h (55 ng, $P < 0.001$), increased further at 4 h at 65 ng ($P < 0.001$), followed by a modest reduction

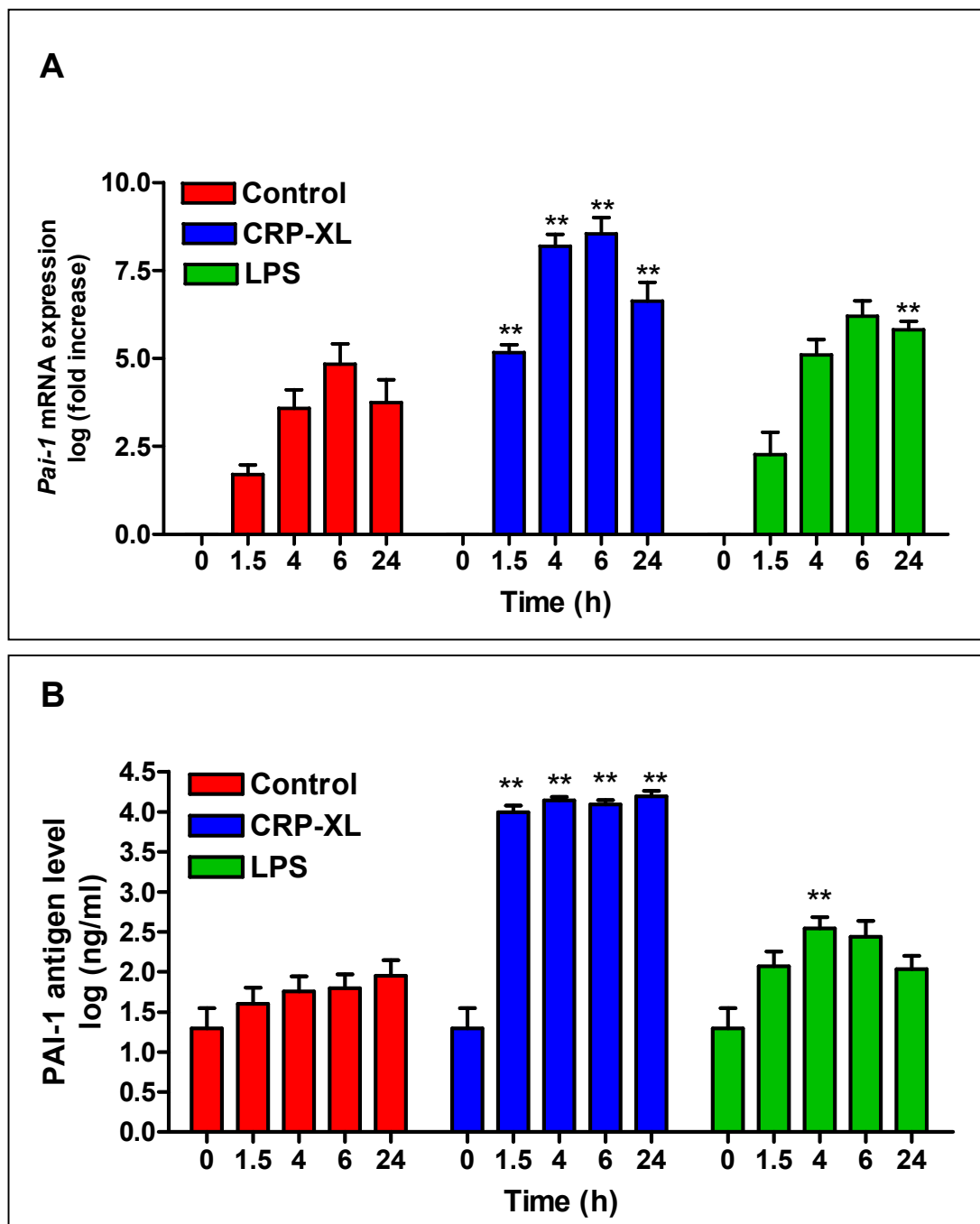


Figure 3.1 – Effect of CRP-XL and LPS on *Pai-1* mRNA and protein expression in monocytes (n=6).

The time course of CRP-XL and LPS induced *Pai-1* mRNA (A) and protein (B) expression determined by real time PCR. Values were normalised to *18s*, expressed as mean \pm SEM fold over baseline (time 0) and log transformed. Both the induction of *Pai-1* mRNA and protein is dependent on time and agonist. ** $P < 0.01$ vs. unstimulated monocytes (control). Two-way ANOVA was used for statistical analysis.

at 6 hrs (60ng, $P < 0.001$), and slight increase after 24 hrs (67ng, $P < 0.001$). PAI-1 antigen levels only significantly differed at 6 h (13 ng, $P < 0.01$) in LPS stimulated samples.

3.2.2 – Platelet-induced PAI-1 in monocytes does not require cell-cell contact

To investigate if platelets could up-regulate monocyte PAI-1 expression without direct cell-cell binding, *Pai-1* expression was examined in monocyte taken from whole blood that was stimulated with CRP-XL in the presence or absence of Mab 9E1, which inhibits P-selectin binding to P-selectin glycoprotein ligand-1 (PSGL1), hence suppressing the binding between platelets and monocytes. The final concentration of Mab 9E1 used (25 $\mu\text{g/mL}$) had been shown to abolish monocyte platelet aggregate and the 4h time point was chosen because, as demonstrated previously, maximal *Pai-1* induction was seen at this time. The percentage of monocytes with adherent platelets was determined by flow cytometry performed in whole blood. Events positive for both CD14 and CD42b demonstrated monocyte platelet aggregate and single monocytes were shown as CD14 positive but CD42b-negative events. For logistical reasons, the flow cytometry analyses were performed by Tina James (Department of Cardiovascular Sciences).

Mixing monocytes with platelets activated by CRP-XL resulted in platelets binding to $\geq 70\%$ of monocytes (Fig. 3.2, ANOVA for Treatment $F_{(2,12)} = 29.27$, $P = 0.0008$; for Time $F_{(2,12)} = 28.14$, $P < 0.0001$; for TM x T interaction $F_{(4,12)} = 48.66$, $P < 0.0001$). This tethering of monocytes and platelets was abolished by the addition of Mab

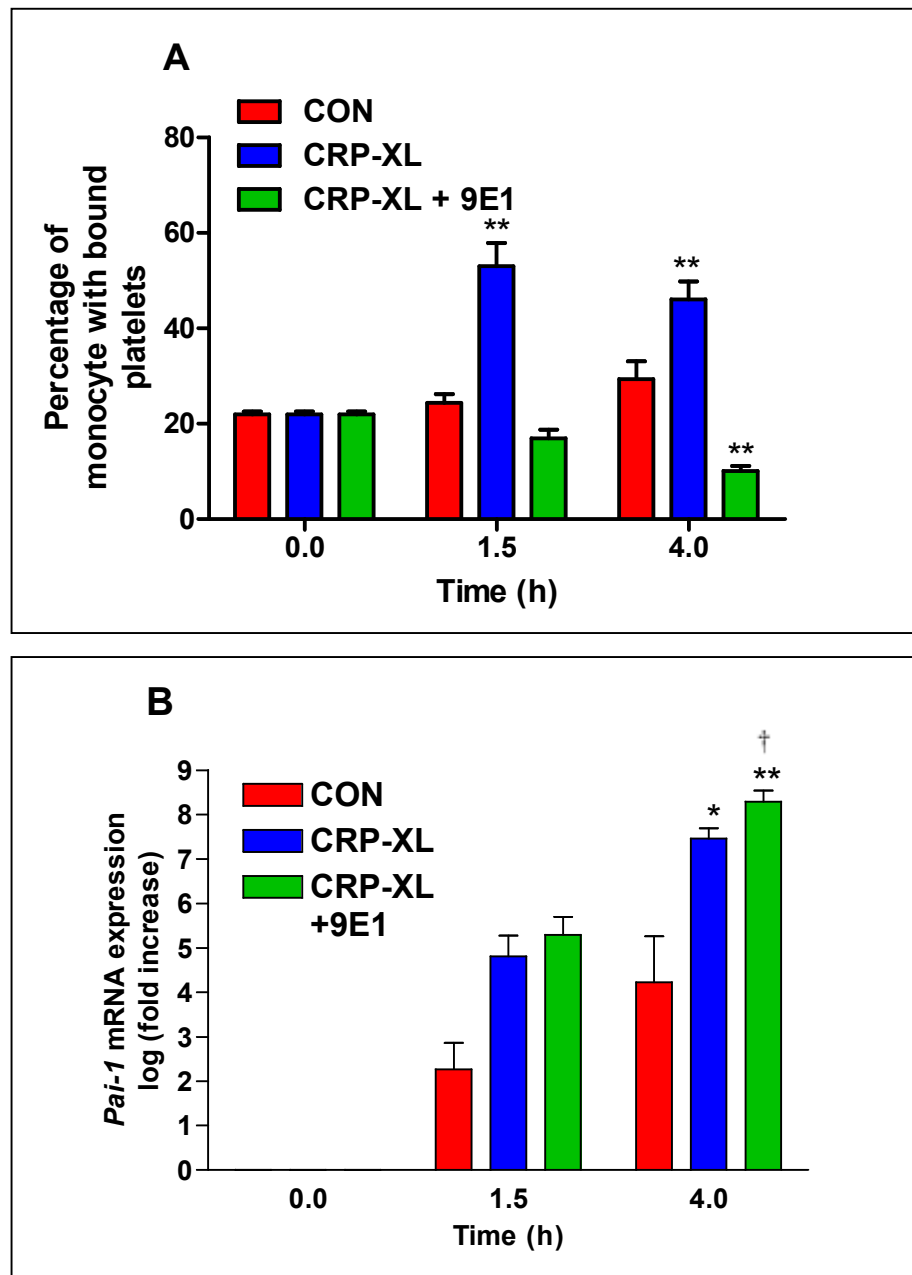


Figure 3.2 – Prevention of platelet-monocyte aggregate resulted in increased up-regulation of *Pai-1* expression in monocytes (n=3).

Whole blood was incubated with CRP-XL in presence or absence of Mab 9E1 1.5-4 h., which inhibited the interaction of P-selectin and PSGL-1. Mab 9E1 prevented the adherence of platelets to monocytes, as shown by flow cytometry, values are shown as mean \pm SEM and arcsin transformed (A). *Pai-1* mRNA expression determined by real time PCR, values were normalised with *18s*, expressed as mean \pm SEM and log transformed (B). * $P < 0.05$ and ** $P < 0.001$ vs. unstimulated monocytes (control), † $P < 0.05$ vs. CRP-XL alone. Two-way ANOVA was used for statistical analysis.

9E1. As found previously, monocyte *Pai-1* expression was induced significantly after 4 h ($P < 0.05$) stimulation with CRP-XL (Fig. 3.2, Agonist ($F_{(2,12)} = 8.58$, $P = 0.0174$), Time ($F_{(4,12)} = 9.14$, $P = 0.0013$). Furthermore, this induction was further enhanced in the presence of Mab 9E1 ($P < 0.01$ at 4 h). These results demonstrate that there is a positive regulation of monocyte PAI-1 by platelets, not requiring cell-cell contact, and that the PSGL-1 signal may be inhibitory.

3.2.3 – Platelet releasate stimulates monocyte *Pai-1*

To explore the importance of soluble mediators in the regulation of monocyte *Pai-1*, platelets were activated by incubation with 50µg/mL of CRP-XL at 37°C for 10 min. The activation time of 10 min was chosen because as shown in later chapter, most of PAI-1 and presumably other soluble mediators, were released by this time. After incubation, the platelet releasate was collected and used to treat isolated MNC preparations for 4 h at 37°C. Monocytes were then isolated and measured for *Pai-1* expression. As shown in figure 3.3, the platelet releasate promoted significant accumulation of *Pai-1* transcript after 4 h of incubation. This is additional evidence that products rapidly released by platelet degranulation provide a sufficient signal to up-regulate monocyte *Pai-1* expression.

3.2.4 – Role of TGF-β1 secreted by platelets in monocyte *Pai-1* induction

Growth factors and cytokines are stored in considerable amounts in platelet α-granules, and are released following platelet activation. The contribution of individual mediators

released from granules was assessed by the use of inhibitors that target specific signalling pathways. Releasates were incubated with neutralising antibodies to TGF- β 1 and interleukin-1 β (IL-1 β) for 10mins at a final concentration of 100 μ g/mL and 1 μ g/mL respectively. The concentration of both antibodies used had been shown by the

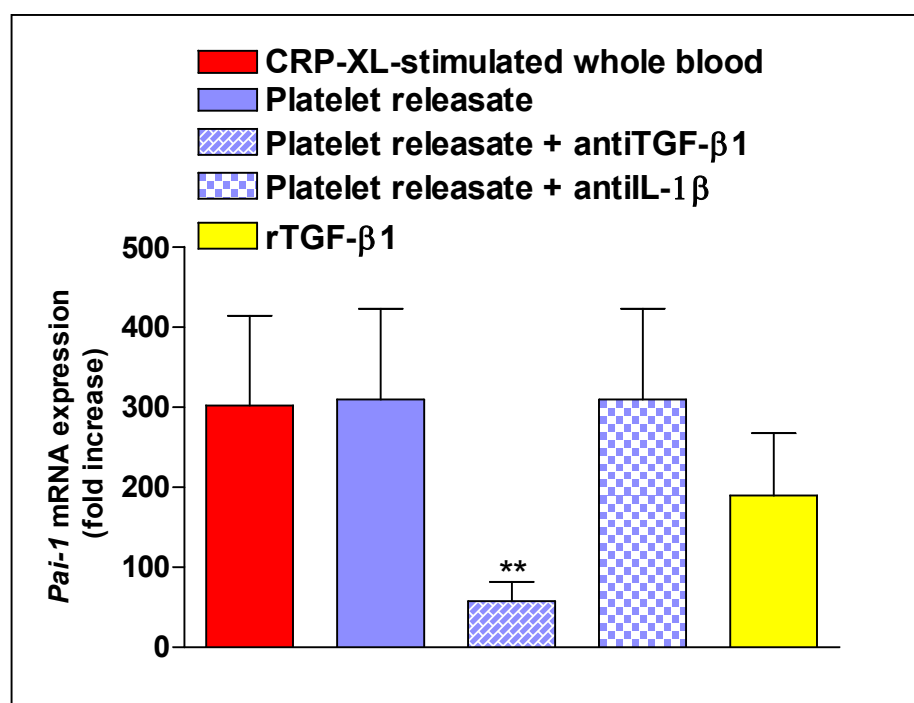


Figure 3.3 – TGF- β 1 released by platelets mediates *Pai-1* mRNA in monocytes (n=6).

Neutralising antibodies to IL-1 β and TGF- β 1 were incubated with platelets releasates stimulated with CRP-XL. Monocytes were then exposed to this pre-treated platelets releasates. *Pai-1* mRNA expression determined by real time PCR. Values were normalised with *18s* and expressed as mean \pm SEM. ** P < 0.01 vs. platelet releasate. One-way ANOVA was used for statistical analysis.

manufacturer to inhibit TGF- β 1 and IL-1 β at the concentrations present in platelets. The platelet releasate was then subsequently added to monocytes for 4 h at 37°C (Fig. 3.3).

Blocking mAb to IL-1 β did not affect *Pai-1* transcript levels.

In contrast, incubation of monocytes with blocking mAb to TGF- β 1 significantly reduced *Pai-1* expression by >80% ($P < 0.01$). These results suggest TGF- β 1 is the principle mediator for platelet-induced monocyte *Pai-1* expression.

To determine if TGF- β 1 alone is sufficient to induce monocyte *Pai-1* expression, monocytes were exposed to recombinant TGF- β 1 (rTGF- β 1) for 4h at 37°C (Fig. 3.3). Final concentration of rTGF- β 1 used was 0.4 ng/ml, and the manufacture demonstrated that this concentration rTGF- β 1 exerts is greatest activity. It was found that rTGF- β 1 increased *Pai-1* expression in monocytes, albeit at a lower level as that seen in monocytes incubated with platelet releasates.

Taken together, the data suggest that TGF- β 1 can alone cause the induction of monocyte *Pai-1* expression, but may act in concert with other mediators released from activated platelets to exert its full effect.

3.2.5 – P-selectin directly induces *Pai-1* expression mediated by the release of TGF- β 1

Although blocking the binding of platelet P-selectin to monocyte PSGL-1 did not inhibit platelet-induced monocyte *Pai-1* expression, whether P-selectin can initiate

monocyte *Pai-1* expression was examined. Monocytes were incubated for 4 h with 20 µg/mL recombinant P-selectin fused to the Fc region of human IgG1 (rP-Selectin/Fc chimera; Fig. 3.4). Peak activity was shown by the manufacture of rP-selectin at this concentration. P-selectin acts by binding to PSGL-1 expressed on the surface of monocytes. It was found that *Pai-1* expression increased in monocytes exposed to rP-Selectin/Fc chimera, although, previously seen with rTGF-β1, induction of *Pai-1* expression by rP-Selectin, the level was significantly lower than when monocytes with stimulated with platelet releasate ($P < 0.01$). These experiments indicate that P-Selectin alone is sufficient to induce monocyte *Pai-1* expression, but the platelet must also present one or more signalling molecules to elicit the full response.

To investigate the mechanism responsible for the induction of *Pai-1* expression in monocyte by P-Selectin, MNCs were incubated with 20 µg/mL of rP-Selectin together with the neutralizing antibody to TGF-β1 at a concentration of 100 µg/mL (Fig. 3.4). It was found that the blocking mAb to TGF-β1 significantly inhibited P-Selectin-induced monocyte *Pai-1* expression ($P < 0.01$), suggesting that the up-regulation of monocyte PAI-1 expression by P-selectin is predominantly via TGF-β1, released by the monocytes via activation through PSGL-1 signalling.

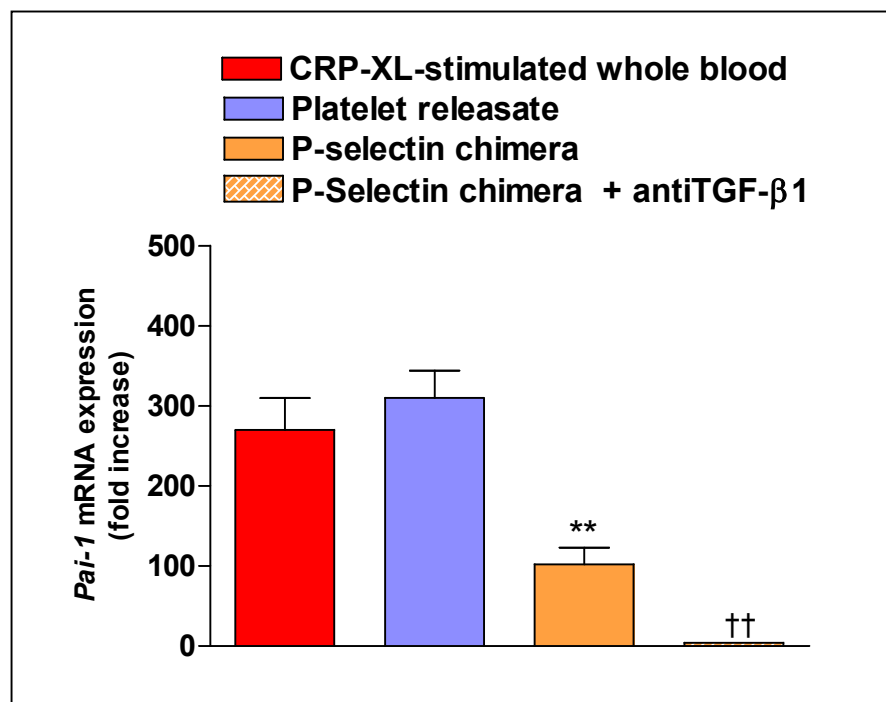


Figure 3.4 – Interaction of P-selectin and PSGL1 directly induce *Pai-1* mRNA in monocytes, regulated by TGF-β1 (n=6).

rP-Selectin/Fc chimera alone increased *Pai-1* expression in monocytes, and attenuated with the co-incubation of neutralising antibody TGF-β1. *Pai-1* mRNA expression determined by real time PCR Values were normalised with *18s* and expressed as mean ± SEM. ** $P < 0.01$ vs platelet releasate and †† $P < 0.01$ vs. P-selectin chimera. One-way ANOVA was used for statistical analysis.

3.2.6 – Incubation of isolated monocytes for 4 h was sufficient to attenuate *Tgf-β1* gene expression

To examine if the binding or soluble mediators released by activated platelets induced monocyte *Tgf-β1* mRNA expression, monocytes stimulated by platelet releasates and P-selectin chimera were utilised (section 3.2.3 and 3.2.5). It was observed that the incubation of monocytes with PBS alone was sufficient to inhibit *Tgf-β1* gene expression (Fig. 3.5, $P < 0.01$). Similar down regulation was seen in the presence of platelet releasates ($P < 0.01$) or P-selectin chimera ($P < 0.01$) on the monocyte *Tgf-β1* gene. These results suggest that the primary level of control may not be the regulation of expression of *Tgf-β1* mRNA, but in the regulation of both the secretion and activation on latent forms of TGF-β1.

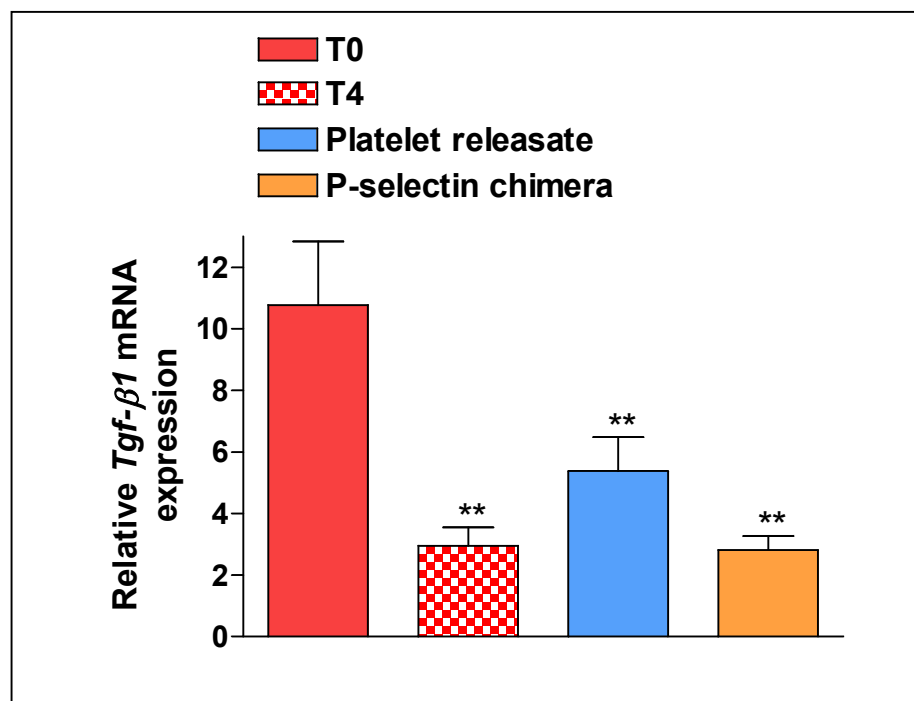


Figure 3.5 – Incubation of monocytes for 4 h was sufficient to down-regulate monocyte *Tgf-β1* expression (n=6).

The incubation of monocyte with PBS buffer for 4 h (T4) was sufficient down-regulate *Tgf-β1* expression. Soluble mediators released by CRP-XL activated platelets and P-selectin chimera alone did not influence the suppression of *Tgf-β1* expression. *Tgf-β1* mRNA expression determined by real time PCR Values were normalised with *18s* and expressed as mean \pm SEM. ** $P < 0.01$ vs. T0 (monocytes at basal conditions). One-way ANOVA was used for statistical analysis.

3.2.7 – Genotyping of the PAI-1 promoter

DNA was isolated from blood cells taken from healthy volunteers. PCR reactions were set up using the genomic DNA as template to amplify the *Pai-1* promoter region. The PCR products were purified and then sequenced using the automated sequencing facility at the Protein and Nucleic Acid Chemistry Laboratory located in the University of Leicester. The resulting chromatogram was then analyzed to determine the genotype of the healthy volunteers (Fig. 3.6). In the chromatogram, *4G/4G* homozygous subjects were identified when four guanine bases were located in the promoter polymorphism site. Four guanine bases with an overlapping adenine base at the end, and five guanine bases were recognized as *4G/5G* heterozygotes and *5G/5G* homozygous genotype respectively. Of the 22 people who were genotyped, 10 were *4G/4G* homozygous, 11 were heterozygous and 1 was *5G/5G* homozygous. Since only one *5G* homozygous individual was found, it was not viable to carry out experiments on this group, hence further examination in the regulation of *Pai-1* by the polymorphism was carried out on *4G* homozygous and *4G/5G* heterozygous subjects.

3.2.8 – Influence of the polymorphism in whole blood stimulation

The induction of monocyte *Pai-1* in whole blood stimulation (as described in section 2.3.1) was analysed for the influence of the *4G/5G* polymorphism. The level of PAI-1 in stimulated whole blood was calculated by taking away the contribution made by plasma. The expression of *Pai-1* mRNA did not differ significantly between the

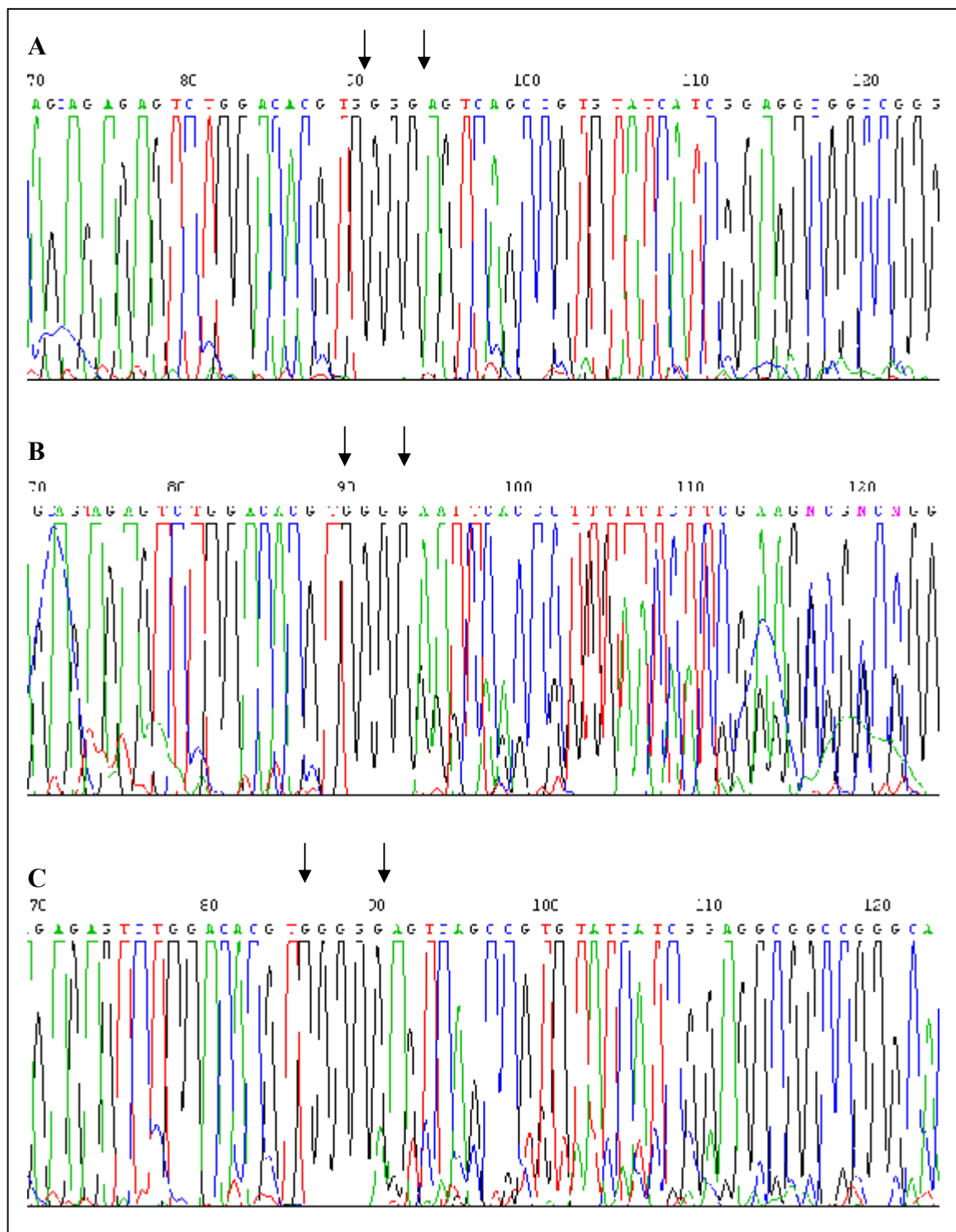


Figure 3.6 – Chromatograms of genotyping

Healthy volunteers were genotyped for the 4G/5G *Pai-1* polymorphism. The black arrows indicate the site at which the polymorphism is located. From studying the chromatograms, each volunteer was assigned as either 4G/4G genotype (panel A), 4G/5G genotype (panel B) or 5G/5G genotype (panel C).

genotypes in unstimulated monocytes at any time point (Fig 3.7, Genotype $F_{(1,16)} = 0.25$, *ns*; Time $F_{(4,12)} = 8.58$, $P < 0.0001$). This was also true for monocytes stimulated with CRP-XL (Genotype $F_{(1,16)} = 0.11$, *ns*; Time $F_{(4,16)} = 99.77$, $P < 0.0001$) or LPS (Genotype $F_{(1,16)} = 0.53$, *ns*; Time $F_{(4,16)} = 90.92$, $P < 0.0001$). By contrast, the level of PAI-1 antigen in the plasma from *4G* homozygous subjects was found to be greater than that seen in heterozygous subjects, (Fig. 3.8, Genotype $F_{(1,32)} = 41.48$, $P = 0.0002$; Time $F_{(4,32)} = 18.38$, $P < 0.0001$). This significant genotype difference was also observed in whole blood stimulated with LPS (Genotype $F_{(1,32)} = 8.29$, $P = 0.0206$; Time $F_{(4,32)} = 31.34$, $P < 0.0001$) and CRP-XL (Genotype $F_{(1,32)} = 5.55$, $P = 0.0463$; Time $F_{(4,32)} = 437.91$, $P < 0.0001$). It therefore appears that the *4G/5G* polymorphism regulates the production of PAI-1 by monocytes, but not its mRNA expression.

To test whether the induction of monocyte *Pai-1* by the platelet secretome is dependent of the promoter polymorphism, the experiment performed in section 3.2.3 was examined in relation to the genotype (Fig. 3.9). Monocytes from *4G/4G* heterozygous subjects expressed higher *Pai-1* levels than *4G/5G* homozygous subjects (180-fold and 151-fold respectively), however the difference was not statically significant. The influence of the polymorphism on specific mechanism of induction of monocyte *Pai-1* was also investigated. Experiments carried out on the effect of rTGF- β 1 and rP-selectin (as described in section 3.2.4 and 3.2.5 respectively) were also analysed in relation to the polymorphism. As seen previously for platelet releasate, *Pai-1* expression was found to be elevated in monocytes originating from *4G* homozygous volunteers in comparison

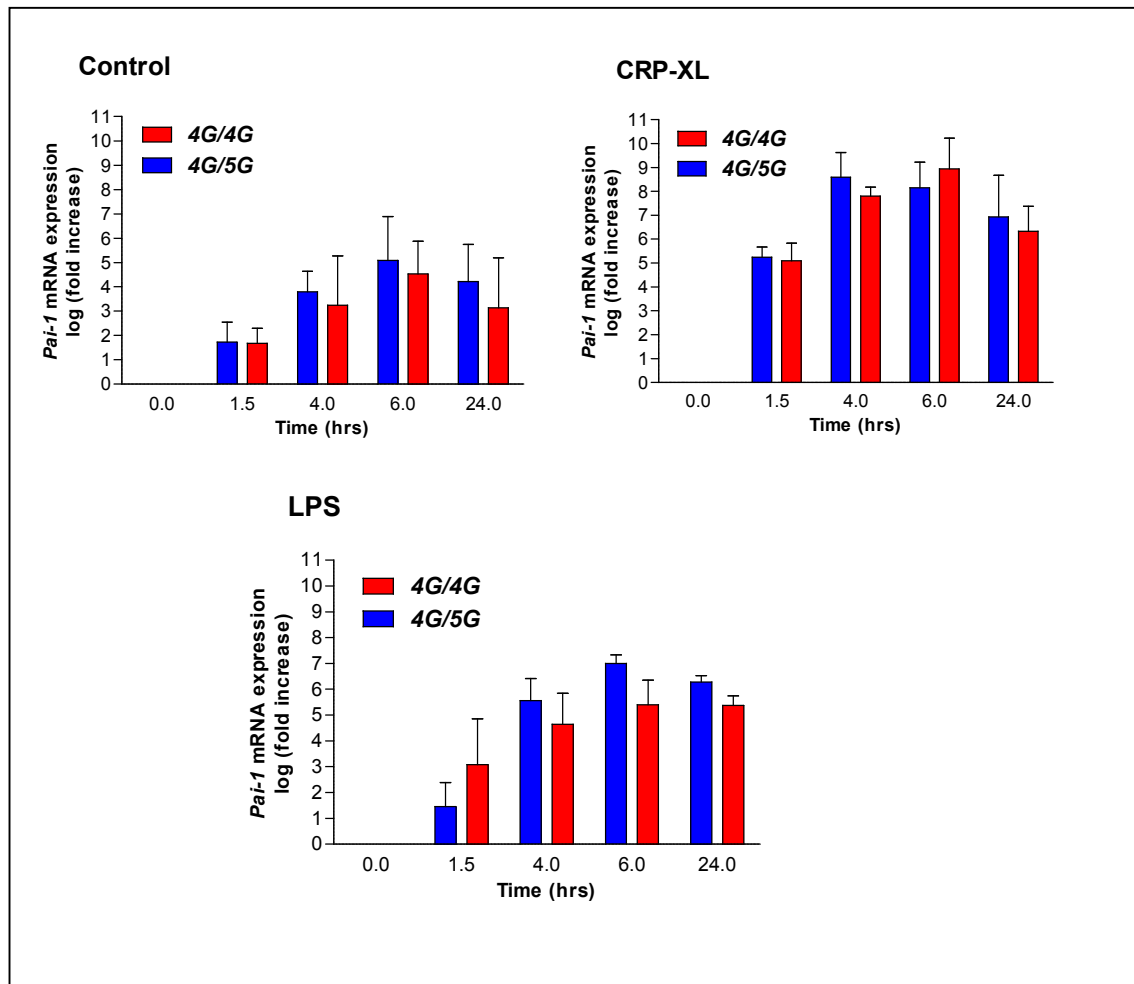


Figure 3.7 – Effect of genotype on *Pai-1* mRNA expression in whole blood (n=6, 3 of each genotype).

The experiments carried out for the induction of whole blood was re-analysed to examine the influence of PAI-1 genotype on monocyte *Pai-1* mRNA expression. None significant difference in *Pai-1* transcript level was observed in unstimulated monocytes, monocyte stimulated by either activated platelets, or LPS at all time points between 4G homozygous (red) and heterozygous subjects (blue). Two-way ANOVA was used for statistical analysis.

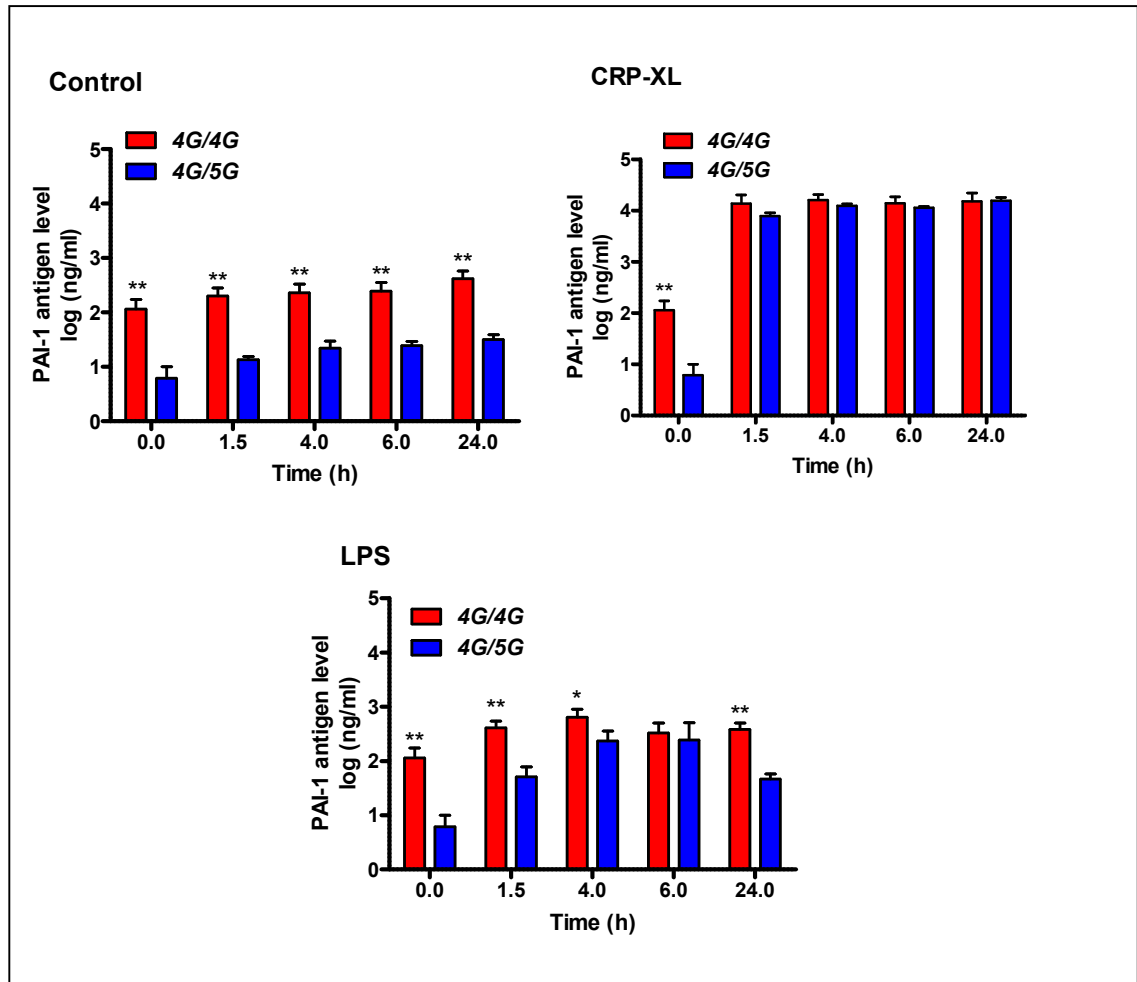


Figure 3.8 – Effect of genotype on PAI-1 protein release in whole blood (n=6, 3 of each genotype).

The experiments carried out for the induction of whole blood was re-analysed to examine the influence of PAI-1 genotype on monocyte PAI-1 protein expression. Whole blood originating from 4G homozygous subjects (red) expressed statistically higher protein levels than heterozygous subjects (blue) in resting conditions. Significant differences in PAI-1 protein level were also observed in whole blood stimulated by either activated platelets, or LPS. Baseline level of PAI-1 was subtracted from stimulated whole blood. * $P < 0.05$ and ** $P < 0.01$ vs heterozygous subjects. Two-way ANOVA was used for statistical analysis.

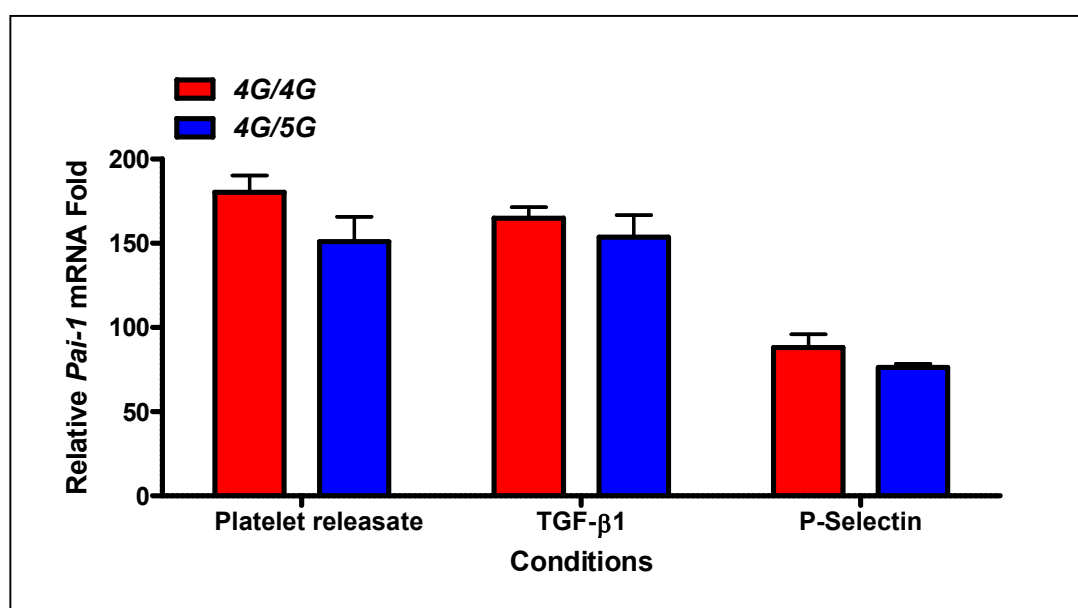


Figure 3.9 – Influence of genotype on the induction of monocyte *Pai-1* by platelets (n=6, 3 of each genotype).

To test the relative contribution of the 4G/5G polymorphism in the induction monocyte *Pai-1* by platelets, monocyte was incubated with releasate secreted from activated platelets, rTGF-β1 and rP-selectin. No detectable difference in *Pai-1* transcript level was observed in all conditions. *Pai-1* mRNA expression determined by real time PCR and values were normalised with *18s*. One-way ANOVA was used for statistical analysis.

to heterozygous volunteers (165-fold compared 153-fold and 88-fold against 76-fold respectively), but not in a statically significant manner. Hence, the promoter polymorphism does not appear to play a role in the regulation of monocyte PAI-1 induced by either platelets or LPS.

3.2.9 – Diurnal variation in plasma PAI-1

To confirm that plasma PAI-1 displays time and genotype-dependent variation, whole blood was taken from 10 volunteers (five *4G* homozygotes and five heterozygotes) at two time points; 9 am and 4 pm. The time points were chosen based on previous studies demonstrating PAI-1 levels oscillates between these two time points (Andreotti and Kluft 1991). Total PAI-1 antigen levels in plasma were measured by ELISA. ANOVA of data showed that plasma PAI-1 levels exhibited diurnal variation that was associated with the *4G/5G* polymorphism, (Genotype $F_{(1,8)} = 6.39$, $P = 0.0354$; Time $F_{(1,8)} = 13.92$, $P = 0.0058$; T x G interactions $F_{(1,8)} = 10.93$, $P = 0.0108$, suggesting that the type of genotype and time points interact in their effect on monocyte PAI-1 antigen levels), with statically significant genotype differences observed in the morning time point (Fig. 3.10, $P < 0.01$). These results confirm that circulating PAI-1 levels in the donors within the current study displays circadian variation which is related to the *4G/5G* polymorphism.

3.2.10 Evidence for circadian variation of *Pai-1* mRNA in whole blood

Whole blood was taken from 7 subjects, (three *4G/4G* homozygous and four *4G/5G* heterozygous), at 9 am (morning) and 9 pm (night). The time points were chosen in accordance to previously published data on the diurnal rhythms of PAI-1 levels found in plasma. The extraction of blood was carried out on 2 different days to confirm the consistency of the experiment. At each time point, RNA was extracted from the blood cells (carried out by Vervan Codd, Department of Cardiovascular Sciences). Expression of *Pai-1* was investigated using a TaqMan probe for PAI-1 in real-time PCR. Since the circadian oscillations of plasma PAI-1 has been found to be dependent upon the *4G/5G* polymorphism, whole blood data was also examined for the influence of the genotype. Significant genotype effect on *Pai-1* expression in whole blood was detected (Fig. 3.11, Genotype $F_{(1,4)} = 14.69$, $P = 0.0186$; Time $F_{(1,4)} = 10.54$, $P = 0.0315$), which was dependent on time (T x G interactions $F_{(1,4)} = 93.84$, $P = 0.0006$). Elevated levels of *Pai-1* expression was observed in *4G/4G* homozygous subjects compared to *4G/5G* heterozygous donors in the morning ($P < 0.01$). The effects of genotype on *Pai-1* expression appear to dampen in the evening time point. These data indicate that *Pai-1* expression in blood cells under basal conditions is regulated by both the circadian clock and the *4G/5G* polymorphism.

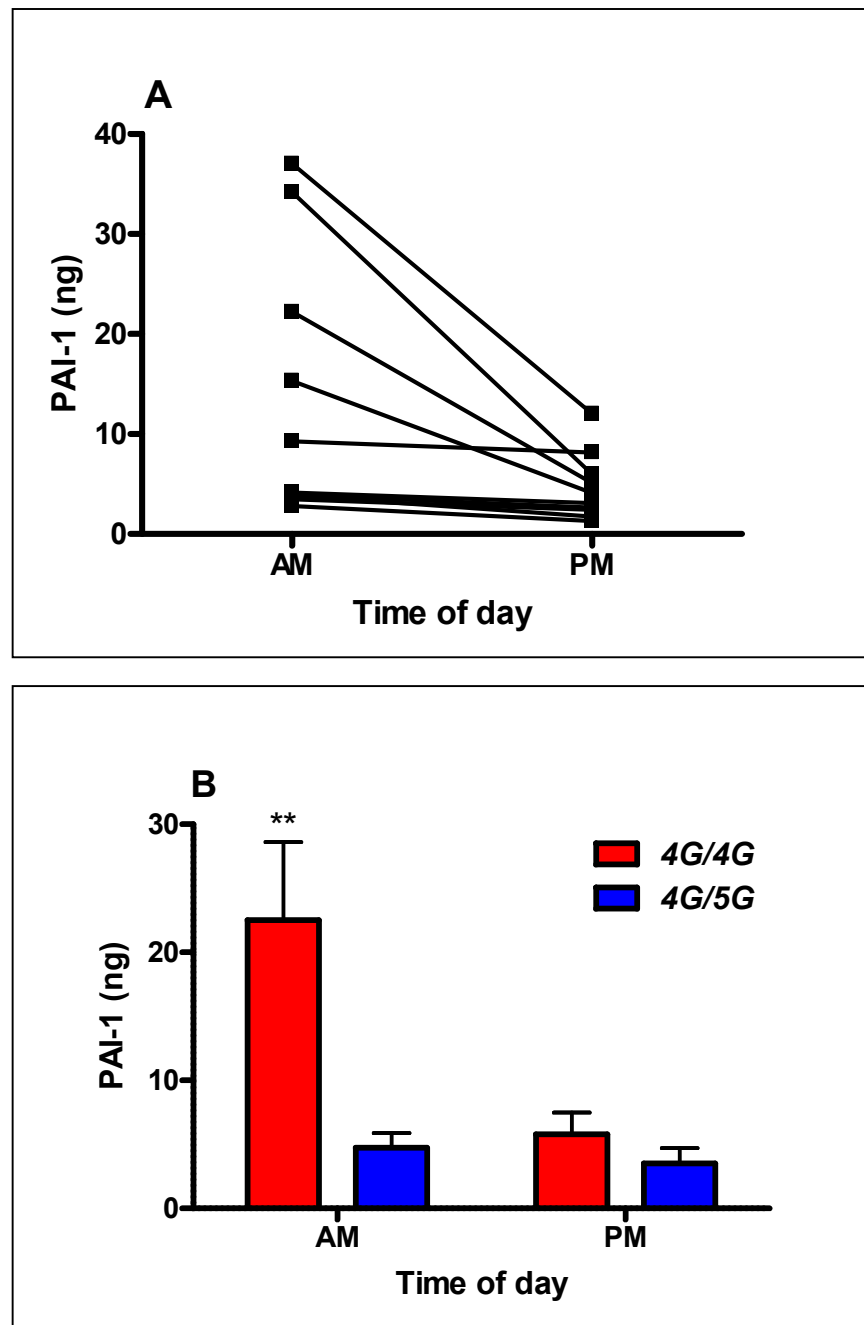


Figure 3.10 – Diurnal variation in plasma PAI-1 (n=10, 5 of each genotype).

Whole blood was taken from 10 volunteers at 9 am (AM) and 4 pm (PM), 5 of *4G/4G* homozygous (red) and 5 of heterozygous origin (blue). (A) Variation in plasma PAI-1 levels between morning and evening time points are shown for each subject, values expressed as mean \pm SEM. (B) PAI-1 levels in the plasma display diurnal variation which was dependent upon the *4G/5G* polymorphism. ** $P < 0.01$ vs heterozygous subjects. Two-way ANOVA was used for statistical analysis.

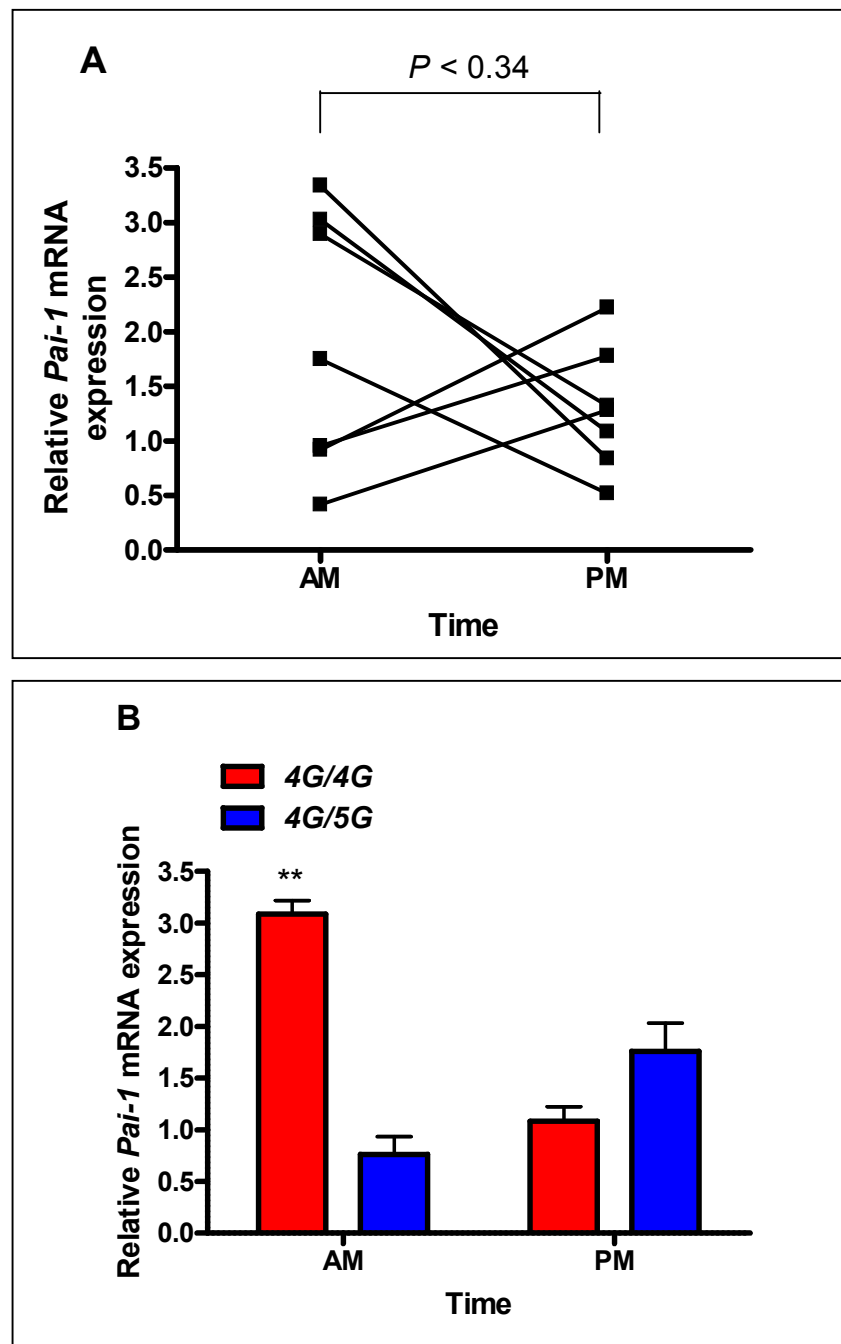


Figure 3.11 – Expression of *Pai-1* in whole blood is influenced by both the circadian clock and promoter polymorphism (n=7, 4G/4G = 3, 4G/5G = 4).

Whole blood was taken from three 4G homozygous (red) and four heterozygous subjects (blue) at 9 am (AM) and 9 pm (PM). (A) Variation in *Pai-1* expression between morning and evening time points are shown for each subject, with values expressed as mean \pm SEM. (B) The abundance of *Pai-1* transcript displayed a genotype dependent diurnal variation. *Pai-1* mRNA expression determined by real time PCR. Values were normalised with *18s*. * $P < 0.01$ vs heterozygous subjects. Two-way ANOVA was used for statistical analysis.

3.3 – Discussion

3.3.1 – Induction of monocyte *Pai-1* expression following platelet activation

In respect of the vasculature, previous reports have reported and focused on the up-regulation of PAI-1 by endothelial cells. To our knowledge, this is the first report to demonstrate the ability of activated platelets to induce PAI-1 expression in human monocytes. The present study demonstrated that monocyte PAI-1 expression was only rapidly induced by activated platelets stimulated by CRP-XL or through GPVI receptors. The time course of *Pai-1* mRNA and protein induction was transient, peaking at 4 h and declined when incubation time was prolonged to 24 h. These data suggest that the newly induced inhibitor equips monocytes for an immediate response that is self-limiting in time. This temporal profile is similar to that reported for *Cyclo-oxygenase-2* (*Cox-2*) expression in monocytes stimulated by activated platelets (Eligini *et al.* 2007). CRP-XL is a synthetic peptide that mimics the triple-helical structure of collagen and, when stabilized by cross-linking, activate platelets through the glycoprotein VI (GPVI) receptor (Morton *et al.* 1995), but does not activate monocytes on its own. LPS (lipopolysaccharide from gram negative bacteria) on the other hand represents an inflammatory stimulus (present during infections like sepsis) that acts through a Toll-like receptor 4 (TLR4) on the monocyte surface. It was shown that *Pai-1* expression was minimally affected by LPS, however protein expression was up-regulated. Previous reports have shown LPS to negatively regulate PAI-1 both at mRNA and protein levels (Lungren *et al.* 1994; Eggesbo *et al.* 1995; Ritchie *et al.* 1995). The discrepancy may be attributed to the advances in the different techniques, as the sensitivity of the methods

has increased over time. In addition, this study examined monocytes in whole blood rather than in isolated preparations. The data demonstrate that expression of *Pai-1* mRNA and PAI-1 protein by monocytes is much more responsive to a thrombotic stimulus compared to an inflammatory stimulus, which is confirmed by Farruggia *et al.* (Farruggia *et al.*, *Blood*, in press) using the same experimental method but analysis was carried out using Illumina genome-wide-arrays. These data suggest that the PAI-1 produced by monocytes may play an important role in thrombosis, possibly in stabilising the developing thrombus.

3.3.2 – Role of TGF- β 1 and P-selectin in the regulation of monocyte PAI-1 by platelets

In this study, a mechanism is described through which platelets regulate monocyte PAI-1 production. The importance of soluble mediators released by activated platelets in this induction was highlighted since blocking the attachment of platelets to monocytes via P-selectin did not inhibit the induction of PAI-1 in monocytes. In fact, blocking platelet-monocyte aggregation appears to lead to increased *Pai-1* expression, which would suggest that direct interaction has an inhibitory effect. Cell to cell contact has been shown as essential for chemokine synthesis and *Cox-2* expression in platelet-monocyte aggregates (Weyrich *et al.* 1996; Dixon *et al.* 2006). In this report, an increase in PAI-1 expression was observed when monocytes were exposed to releasates of activated platelets, without the requirement of cell to cell contact. This positive paracrine regulation, not requiring cell-cell contact, has also been observed in other report for *Cox-2* regulation (Eligini *et al.* 2007).

Platelet secretome is made up of >300 different type of proteins (Coppinger *et al.* 2004), of the possible soluble mediators, two were chosen which have been demonstrated to regulate PAI-1 levels. Since IL-1 β has been shown to induce PAI-1, and this cytokine is reportedly synthesised by thrombin-activated platelets, it was anticipated that this would play a role when platelets were activated by CRP-XL. TGF- β 1 is present in large amounts in α -granules of platelets and is the richest source of TGF- β 1 in humans (Assoian *et al.* 1983). PAI-1 has been demonstrated to be up-regulated by TGF- β 1 in a variety of cell types (Fitzpatrick and Graham 1998; Buisson *et al.* 1998; Dong-Le Bourhis *et al.* 1998; Treichel *et al.* 1998), but most notably in endothelial cells both *in vitro* and *in vivo* (Saksela *et al.* 1987; Dong *et al.* 1996) hence may also function to regulate monocyte PAI-1. Neutralising antibodies to TGF- β 1 blocked platelet-induced *Pai-1* expression by >80% ($P < 0.01$), but antibodies to IL-1 β had no effect, leading to the hypothesis that TGF- β 1 is the physiologically relevant mediator of PAI-1 induction by activated platelets. This hypothesis was supported by the finding that rTGF- β 1 alone induced monocyte *Pai-1* expression. However, rTGF- β 1 induced monocyte *Pai-1* expression was less apparent than platelet releasates, suggesting that either the concentration rTGF- β 1 was insufficient or that mechanisms may contribute to increase *Pai-1* expression in monocytes exposed to releasates.

P-selectin presented by platelets not only mediates cell-cell contact but modifies, and may directly induce, functional responses in monocytes. This was evident when rP-selectin was observed to directly induced monocyte *Pai-1* expression. Here, rP-selectin may induce the secretion of TGF- β 1 by monocytes, which in turn act to up-regulate *Pai-1* expression in an autocrine system. This was demonstrated when monocyte *Pai-1*

expression was significantly attenuated by the neutralising antibody to TGF- β 1, before the monocytes were exposed to rP-selectin. Nagata and co-workers (1994, 1993) reported that thrombin-stimulated platelets cause superoxide anion generation by neutrophils and monocytes, and Yeo *et al.* (1994) found that neutrophils shed L-selectin, upregulate MAC-1, and polarize when they adhere to activated platelets. In these reports the leukocyte responses were inhibited by antibodies directed against P-selectin. This is the first study to show that blocking the interaction of activated platelets to monocyte via P-selectin-PSGL1 enhanced monocyte gene expression, suggesting that P-selectin may inhibit monocyte *Pai-1* expression, and therefore have opposite effects to TGF- β 1.

The results so far suggest that TGF- β 1 is involved in mediating monocyte PAI-1 expression in response to the binding of activated platelets. One possible mechanism of increasing local TGF- β 1 levels is that the binding between stimulated platelets and monocytes increases the expression of monocyte *Tgf- β 1*. However, the current data demonstrated the opposite since a down-regulation of *Tgf- β 1* expression was observed in monocytes incubated alone. This result may not be as surprising as first thought, in fact, the down-regulation of monocyte *TGF- β 1* expression without any stimulation suggest the sensitivity of this mechanism. In monocytes under basal conditions, *TGF- β 1* appears to be highly expressed which is consistent with the findings by other groups (Kitamura *et al.* 1996; Letterio *et al.* 1998). High *TGF- β 1* expression would prepare the monocytes for an acute response following stimulation. Once the monocytes are activated, the immediate response is to release available TGF- β 1, and inhibits its production, as suggested by the current study. This system allows the creation of a mechanism to prevent the over production of TGF- β 1. This mechanism is widely used

by key genes which are constitutively expressed such as the cardiac specific gene MyoD (Montarras, *et al.* 1989). Since TGF- β 1 is stored in the latent, biological inactive form and released upon stimulation, this leads to the question of how the latent TGF- β 1 released by both platelets and monocytes is converted to the active form in the current model. Analysis of data produced from microarray experiments investigating the gene expression of monocytes stimulated by CRP-XL (Farruggia *et al.*, Blood, in press) identified increased expression of Thrombospondin-1 (TSP-1, *THBS-1*). TSP-1 was markedly up-regulated in monocytes stimulated by activated platelets compared to monocyte stimulated by LPS. This molecule can convert latent TGF- β 1 (released by both platelets and monocytes) to its active confirmation (Lawrence 1996; Ribeiro *et al.* 1999; Schultz *et al.* 1999). Physiologically, an interesting question emerges in regard to why this mechanism of converting latent TGF- β 1 to its active state exists in this context, would it not be faster for the monocyte to produce active TGF- β 1, and by past this mechanism? The possible explanation is that TGF- β 1 regulates so many different physiological functions, a tight regulation is necessary to prevent it from acting on different pathways, and this conformational regulation of TGF- β 1 also ensures that TGF- β 1 released by platelets and monocytes only acts upon cells in the local vicinity.

3.3.3 – Regulation of monocyte PAI-1 by the 4G/5G polymorphism

Of the subjects genotyped, a low prevalence of 5G allele was observed, with just a single volunteer homozygous for the 5G/5G genotyped. Additionally, this only 5G/5G donor had an irregular sleep pattern, making them relatively unsuitable to investigate the influence of the 5G/5G genotype on PAI-1 expression. Therefore experiments

compared *4G/4G* homozygous subjects against *4G/5G* heterozygous subjects. Previous studies have shown that PAI-1 levels in plasma are higher in *4G* homozygous subjects compared to the other 2 genotypes, which have similar levels, suggesting that donors carrying only the one *5G* allele is sufficient to exert the effect of lowering PAI-1 levels. Therefore findings from heterozygous subjects should be similar to that of *5G* homozygous subjects. The donors in this study showed a higher bias for the *4G* allele compared to the *5G* allele, which is not consistent with previous reports showing equal prevalence for the *4G* and *5G* allele in the European population (Matsubara *et al.* 1999; Hermans *et al.* 1999; Loktionov *et al.* 2003). This can be explained by the small number in the sample size.

The extent at which the activated platelets induced monocyte *Pai-1* expression was the same for both *4G/4G* homozygous and *4G/5G* heterozygous subjects in whole blood or isolated monocyte preparations. Previous reports have shown an effect of *4G/5G* polymorphism on PAI-1 production in stimulated endothelial cells (McCormack *et al.* 1998; Roncal *et al.* 2004). As the number of subjects in the investigation was relatively small, the power of the study may be limited. Another possibility is that the *4G/5G* polymorphism is cell specific and only regulates PAI-1 in certain cell types. In contrast, the production of PAI-1 antigen in whole blood was associated with the *4G/5G* polymorphism. The differential regulation of monocyte PAI-1 mRNA and protein expression may be due to the insensitive measure of protein levels in these experiments because of the presence of plasma and platelets, which can contribute to PAI-1 antigen levels.

The production of PAI-1 antigen is not exclusive to monocyte alone in whole blood. Another cell type within this system is also capable of secreting PAI-1, specifically the platelets. The regulation of platelet PAI-1 will be examined in later chapter (section 5), but it is worth noting that platelets contain large amount of PAI-1 within their α -granules, which is released upon activation. Since the platelets are activated in this model system, it is possible that the PAI-1 protein produced in stimulated whole blood may originate from platelets. Hence, the influence if the polymorphism on PAI-1 protein may be regulated through platelets rather than monocytes. However, the secretion of PAI-1 protein by whole blood incubated with LPS would suggest that PAI-1 protein is dependent upon the *4G/5G* polymorphism, as LPS directly stimulates the monocytes without activating the platelets.

The likely scenario is the expression of PAI-1 protein by stimulated monocytes is regulated by the *4G/5G* polymorphism, and this differential regulation is exacerbated in whole blood with contribution from platelets in samples incubated with CRP-XL.

Assuming that the release of PAI-1 protein from stimulated monocytes is dependent on the *4G/5G* polymorphism, and monocyte *Pai-1* expression is independent of the genotype, it becomes intriguing to examine the mechanism involved in the differential regulation of mRNA abundance and protein expression. Analysis of the 3' UTR of *Pai-1* gene reveals conservation that is usually seen in microRNA (miRNA) targets. miRNA are short, single stranded RNAs (~20 nucleotides) processed from ~70 nucleotides, genomically encoded primary transcripts that potently inhibit gene expression from target mRNAs. Although their mechanism of action is unclear, it involves base-pairing between the mRNA 3' UTR and miRNA 5' end, leading to translational inhibition

and/or increased mRNA degradation and/or mRNA sequestration (Nilsen 2007). Computer programmes such as RNAhybrid (Krüger and Rehmsmeier 2006), measure the properties of the *Pai-1* mRNA structure confirm that it could be a possible target of miRNA. The 4G/5G polymorphism maybe associated with miRNA gene, so that miRNA may make mRNA produced by one allele less stable then the other, leading to more/less protein manufactured.

3.3.4 – Regulation of monocyte PAI-1 by the circadian clock

Previously, circadian expression of PAI-1 antigen was documented in human plasma of 4G/4G homozygous subjects, with higher levels during the day than night (Margaglione *et al.* 1998a). Consistent with these observations, the present study demonstrated diurnal variation in circulating PAI-1 antigen and expression of the *Pai-1* gene in unstimulated leukocytes. Since PAI-1 expression has only been reported in monocytes in a leukocyte population, these data indicate that monocyte PAI-1 expression is regulated by the circadian clock. However, whether the expression of monocyte *Pai-1* mRNA is directed by the circadian clock system has not been directly demonstrated. In the present study, it was observed that one donor from the heterozygous group was found to not fit this trend, as the *Pai-1* expression for this volunteer was higher in the morning compared to the evening. The reason for this abnormal expression pattern was revealed from data by others in the lab (Very Codd, personal communication) measuring clock gene expression in the same subjects. This volunteer showed a reverse *mPer2* gene oscillation in comparison to others in the heterozygous group, which may affect the

circadian expression of *Pai-1*. These observations support the idea that PAI-1 in monocytes under resting conditions is under the regulation of the circadian clock system.

3.4 – Conclusion

To conclude, the present study shows that monocytes play a role in thrombosis by the expression of PAI-1. During the progression of a developing thrombus, platelet activation would lead to release of a large number of soluble factors including growth factors and cytokines. These platelet-derived factors can stimulate other blood cells including monocytes. Specifically, TGF- β 1 released by platelets following activation can stimulate monocytes to release TGF- β 1 increasing the local level, and inducing the expression of monocyte PAI-1. In general, the findings in this section support the view that the interaction of monocytes with platelets products within the vasculature may establish a gene expression profile, in order for the cells to co-ordinate response to injury in terms of thrombosis. As a possible clinical application, altering the level of TGF- β 1 local to the site of thrombosis may be a good avenue to lowering the level of PAI-1, thus enhancing fibrinolysis.

The present study has provided evidence that *Pai-1* mRNA in whole blood, under physiological conditions, exhibits diurnal variation which is dependent on the promoter 4G/5G polymorphism. Data in this section suggests that the acute induction of whole blood cells is also regulated by the 4G/5G polymorphism. Since induction monocyte *Pai-1* transcript appear not to be influenced by the polymorphism, other cell types (especially platelets) in whole blood may contribute the genotype differences observed

for PAI-1 antigen. However, if the differential regulation PAI-1 levels by the polymorphism were of monocyte origin, miRNA is a possible regulatory mechanism by affecting the stability of *Pai-1* mRNA.

3.5 – Future Work

As a result of the present study, a number of questions has arisen that could be investigated in future work.

(i) Activity of monocyte PAI-1

In the experiments performed to measure the level of PAI-1 produced by monocytes, the ELISA method used detected total PAI-1 antigen. This was to allow for the comparison of monocyte *Pai-1* transcript and PAI-1 protein expression. However, it is acknowledged that the PAI-1 pool produced by stimulated monocytes, may contain active and latent (bound to either tPA or Vn) forms. The relative contribution of monocyte PAI-1 could be further elucidated with the use of an assay that was specific for active PAI-1.

(ii) Measurement of PAI-1 antigen from isolated monocytes

Data in the present study suggest that PAI-1 produced by monocytes is associated with the 4G/5G polymorphism. To confirm this finding, monocytes could be isolated from whole blood and stimulated with various agonists such as platelet releasate and rTGF-

$\beta 1$. The resulting PAI-1 antigen produced can be detected with ELISA, and analysed for the influence of the polymorphism.

(iii) Mechanism of induction of monocyte PAI-1 by TGF- $\beta 1$

Data from this section concluded TGF- $\beta 1$ is the main mediator of monocyte PAI-1 induction by activated platelets. The detailed mechanism by which TGF- $\beta 1$ up-regulates monocyte PAI-1 remains to be explored and a possible signal could be MAP-kinase as shown previously in another cells. To elucidate the role of MAP-kinase activation in PAI-1 induction by both platelet releasates and TGF- $\beta 1$, monocytes could be pre-incubated with compounds that inhibit ERK1/2, p38 and c-jun N-terminal kinase. These include PD98059, SB203580 and SP600125 respectively. Other kinase that have been demonstrated to be involved in the signalling of PAI-1 gene expression include Rho/Rho-kinase, which could be inhibited with Y-27632.

(iv) TSP-1 levels in the regulation of monocyte PAI-1 expression

The regulation of latent TGF- $\beta 1$ to the active confirmation may play a role in the regulation of monocyte PAI-1 expression. As discussed earlier, a possible protein involved in converting the confirmation of TGF- $\beta 1$ is TSP-1. Since TSP-1 was found to be up-regulated in monocytes stimulated by activated platelets, it may play an important role in the regulation of monocyte PAI-1. This hypothesis could be examined by measuring monocyte TSP-1 protein to confirm that the protein is up-regulated by

activated platelets. Then incubating neutralising antibodies to TSP-1 with platelets releasates, and stimulate monocytes with the resulting mixture.

(v) The link between miRNA and 4G/5G polymorphism

It was observed that regulation of monocyte PAI-1 protein expression appears to be influenced by the polymorphism, but independent for the accumulation of mRNA abundance. A possibility lies with the stability of the mRNA transcript, and one mechanism could be miRNA. The first step would be to find out the identity of miRNA that specifically targets *Pai-1* mRNA. Then HapMap could be utilised to further investigate the possible influence of 4G/5G polymorphism and miRNA on monocyte PAI-1 expression. HapMap is a haplotype map of the human genome, which describes the common patterns of human genetic variations. Associations with 4G/5G polymorphism and haplotype of miRNA gene may be examined using this procedure.

Chapter 4

Peripheral circadian clock in resting and stimulated monocytes

4.1 – Introduction

A significant finding from the previous chapter was the importance of activated platelets in the regulation of monocyte PAI-1 expression. Furthermore, TGF- β 1 released upon platelet degranulation appears to mediate the induction of PAI-1 expression in monocytes. *Pai-1* gene contains three SBE (bound by Smad proteins elements) between two E-box elements. It was demonstrated by Hua *et al.* (1999) that the second E-box (located at -599) was essential for the activity of *Pai-1* promoter in conferring TGF- β induced transcription (discussed in section 1.9.1). Moreover, data from our group (Chong *et al.* 2006) has shown that both E-boxes are important in the transactivation of *Pai-1* promoter by CLOCK:BMAL2 heterodimer. Since Smad proteins can interact with different transcription factors, it is possible that SMADs may interact with the CLOCK:BMAL2 heterodimer at the SBE and E-box sites, and create a circadian-dependent PAI-1 response to TGF- β 1. This section of the report examined whether peripheral circadian clock exists in monocytes, and how biological clock may be influenced in monocytes stimulated through inflammatory or thrombotic stimuli.

4.2 – Results

4.2.1 – The expression of clock genes in monocytes under basal conditions

To examine the mRNA transcript abundance of the core clock genes in unstimulated monocytes, clock gene mRNA expression was measured by semi-quantitative PCR in isolated monocytes under resting conditions.

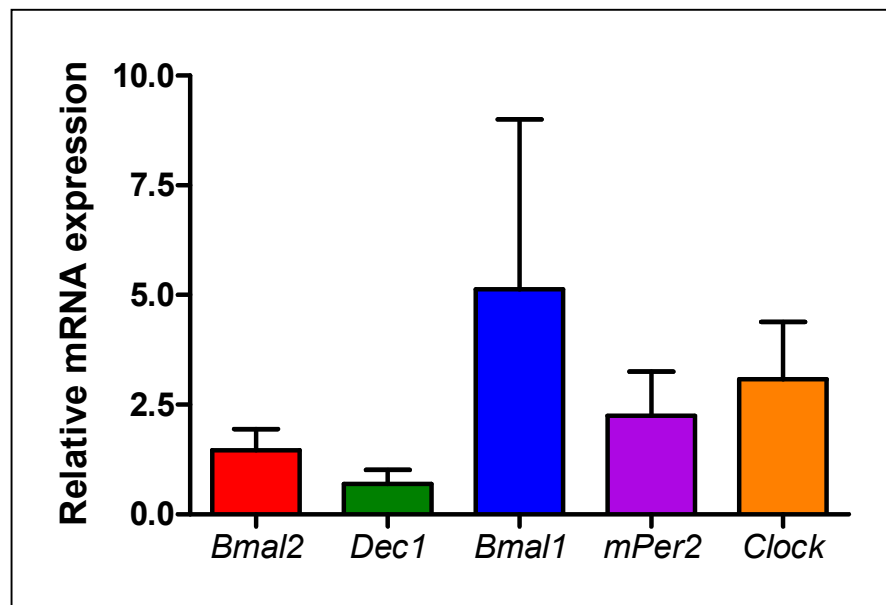


Figure 4.1 – Expression of core clock genes in monocytes under resting conditions (n=8).

The gene expression of *Bmal2* (red), *Dec1* (green), *Bmal1* (blue), *mPer2* (purple) and *Clock* (orange) was measured in isolated monocytes under basal conditions at 9 am, with semi-quantitative PCR. Values were expressed as ratio to *18s* transcript abundance and shown as mean ± SEM.

The relative abundance of the clock gene transcripts are expressed as a ratio to the *18s* control gene (Fig. 4.1). The expression of all clock genes examined was detected in monocytes under basal conditions. *Bmal1* produced the highest mRNA expression (5.2-fold), the least measured was *Dec1* expression (0.7-fold), and the expression of *Clock*, *mPer2*, *Bmal2* fell between these two genes (3.1-fold, 2.2-fold, 1.5-fold respectively).

4.2.2 – The effect of CRP-XL and LPS on the clock gene *Bmal2* expression over time

The expression of *Bmal2* was investigated in monocytes extracted from stimulated whole blood, performed in section 3.2.2 (Fig. 4.2). *Bmal2* mRNA abundance was elevated after 4 h incubation in all samples and declined at 24 h. Levels of expression were low in controls (maximum 1.6-fold at 4 h). CRP-XL did not appear to induce *Bmal2* expression over and above that seen in the unstimulated sample. In contrast, *Bmal2* expression was markedly increased in LPS treated samples, reaching maximum expression after 4 h (18-fold). ANOVA revealed significant Time ($F_{(4,48)} = 6.76$, $P = 0.0002$) and Agonists effect ($F_{(2,48)} = 4.37$, $P = 0.0375$), and T x A interactions ($F_{(8,48)} = 4.50$, $P = 0.0004$), indicating that the type of agonist and time points interact in their effect on monocyte *Bmal2* mRNA levels.

The expression of all the clock genes examined except for *Bmal2* was investigated by semi-quantitative PCR. However, *Bmal2* mRNA abundance was measured by using both semi-quantitative and real time PCR to compare the results produced from the two PCR methods.

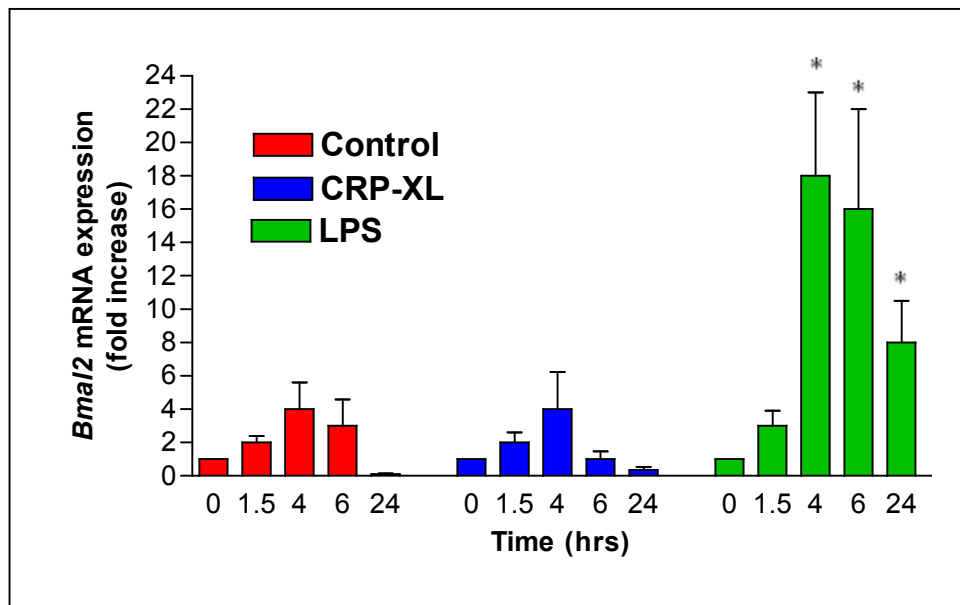


Figure 4.2 – Effect of CRP-XL and LPS on *Bmal2* mRNA expression in monocytes (n=6).

The time course of CRP-XL and LPS induced *Bmal2* mRNA expression determined by real time PCR. Up-regulation of monocyte *Bmal2* expression was seen in LPS stimulated samples (green), whereas CRP-XL (blue) did not appear to have induced *Bmal2* expression over and above that seen in the unstimulated sample (red). Values were normalised with *18s* and expressed as fold over baseline (time 0) with mean \pm SEM. * $P < 0.05$ vs. unstimulated monocytes. Two-way ANOVA was used for statistical analysis.

4.2.3 – Influence of CRP-XL and LPS on Clock gene expression

To examine the effect of LPS on the expression of other core clock genes in monocyte, whole blood was incubated with LPS at 37°C and core clock gene mRNA expression was determined by semi-quantitative PCR in extracted monocytes at time points 0 h, 1.5 h, 4 h. (Fig. 4.3). Of the genes studied only *Dec1* (Agonists $F_{(1,8)} = 0.85$, *ns*; Time $F_{(2,8)} = 44.24$, $P < 0.0001$; T x A interactions $F_{(2,8)} = 10.36$, $P = 0.006$) and *Bmal2* expression (Agonist $F_{(1,8)} = 81.23$, $P = 0.0008$; Time $F_{(2,8)} = 124.55$, $P < 0.0001$; T x A interactions $F_{(2,8)} = 67.82$, $P < 0.0001$) were induced by LPS, demonstrating that LPS and time points interact in their effect on monocyte *Dec1* and *Bmal2* mRNA levels. This observation of *Bmal2* expression is consistent with that seen in real-time PCR (section 4.2.2), indicating the results of semi-quantitative are valid. LPS stimulation produced a statistically significant increase in *Bmal2* mRNA abundance at maximal expression after 4 h (13-fold $P < 0.01$). *Dec1* was also elevated by LPS, becoming significant after 4 h (4-fold, $P < 0.01$). The expressions of *mPer2* (Agonist $F_{(1,8)} = 64.20$, $P = 0.0013$; Time $F_{(2,8)} = 1.83$, *ns*; T x A interactions $F_{(2,8)} = 1.33$, *ns*) and *Clock* (Agonist $F_{(1,8)} = 106.96$, $P = 0.0005$; Time $F_{(2,8)} = 7.04$, $P < 0.0172$; T x A interactions $F_{(2,8)} = 0.57$, *ns*) in monocytes were significantly reduced with the treatment of LPS. After treating monocytes with LPS for 1.5 h, a sharp decrease in *Clock* and *mPer2* mRNA expression could be observed (0.41-fold and 0.31-fold respectively), and levels remained below baseline after 4 h. The addition of LPS to monocytes appear to have a insignificant effect on *Bmal1* mRNA expression

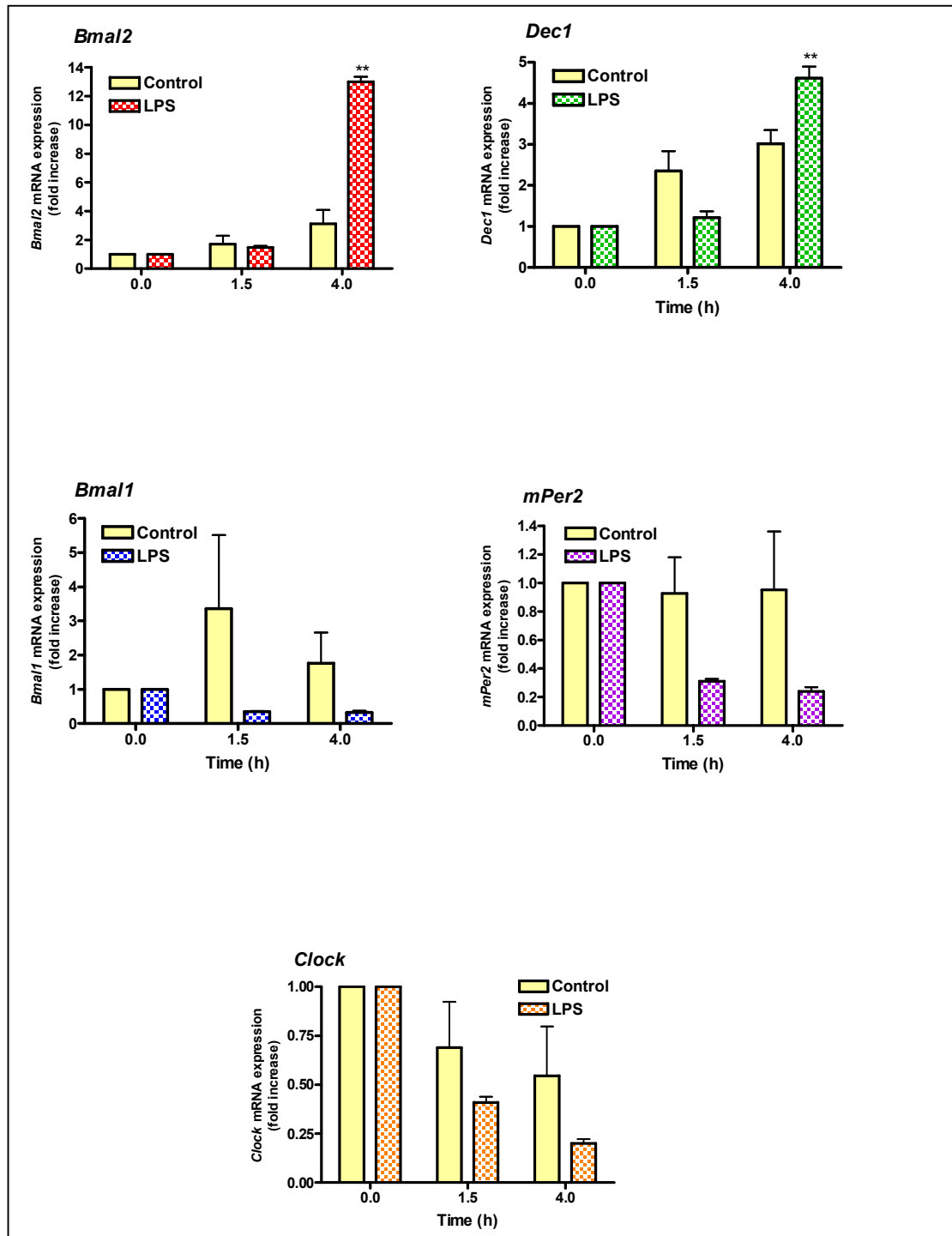


Figure 4.3 – Effect of LPS on clock genes mRNA expression in monocytes (n=6). The time course for the expression of monocyte clock gene in response to LPS was determined by semi-quantitative PCR. The abundance of *Bmal2* (red) and *Dec1* (green) transcript increased in monocytes when incubated with LPS, the expression of *mPer2* (purple) and *Clock* (orange) was down-regulated, and *Bmal1* (blue) expression was not affected. Values were normalised with *18s* and expressed as fold over baseline (time 0) and shown as mean \pm SEM. ** $P < 0.01$ vs. unstimulated monocytes (control). Two-way ANOVA was used for statistical analysis.

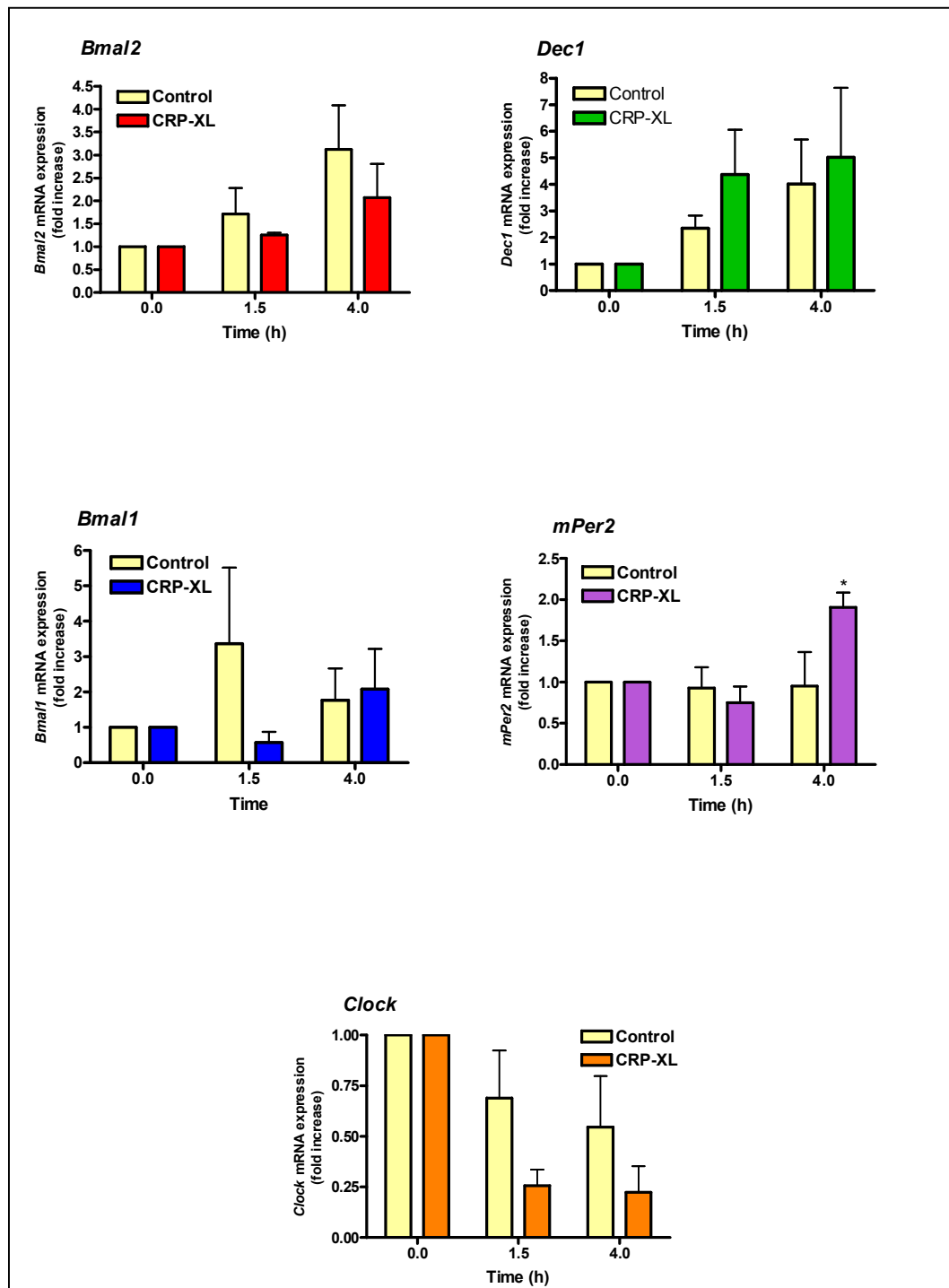


Figure 4.4 – Effect of CRP-XL on monocytes clock genes expression (n=6).

The time course for the expression of monocyte clock gene in response to CRP-XL was determined by semi-quantitative PCR. Incubating monocytes with activated platelets resulted in the up-regulation of *mPer2* expression (purple), and the down-regulation of *Clock* (orange). The other clock genes examined *Bmal2* (red), *Dec1* (green), *Bmal1* (blue) was unaffected by stimulated platelets. Values were normalised with *18s* and expressed as fold over baseline (time 0) and shown as mean \pm SEM. * $P < 0.05$ vs unstimulated monocytes (controls). Two-way ANOVA was used for statistical analysis.

The mRNA abundance of monocyte *Bmal1*, *Bmal2* and *Dec1* appeared to be unaffected by the stimulation of activated platelets (Fig. 4.4). The activation of monocytes via a thrombotic stimulus caused a statically significant up-regulation of *mPer2* expression (Agonist $F_{(1,8)} = 23.65$, $P = 0.0083$; Time $F_{(2,8)} = 2.53$, ns ; T x A interactions $F_{(2,8)} = 2.53$, ns) and down-regulation of *Clock* expression (Agonist $F_{(1,8)} = 158.22$, $P = 0.0002$; Time $F_{(2,8)} = 6.30$, $P = 0.0227$; T x A interactions $F_{(2,8)} = 0.72$, ns). Following stimulation of activated platelets, monocyte *Clock* expression acutely declined at 1.5 h (0.25-fold) and maintained at this level after 4 h (0.25-fold). A summary of clock gene profile in monocyte in response to LPS or CRP-XL can be found in table 4.1.

	LPS	CRP-XL
<i>Bmal1</i>	→	→
<i>Bmal2</i>	↑	→
<i>Dec1</i>	↑	→
<i>mPer2</i>	↓	↑
<i>Clock</i>	↓	↓

Table 4.1 – Summary of the clock gene expression in stimulated monocytes

Profile of clock gene examined in monocytes stimulated with LPS or platelets activated by CRP-XL. An arrow pointing up depicts up-regulation, and an arrow pointing down symbolises down-regulation. Arrow pointing to the right in the table refers to no significant change in clock gene expression.

4.2.4 – Platelet releasate stimulates monocyte *mPer2*

To test the hypothesis that soluble mediators released by activated platelets are important in the induction of monocyte *mPer2* expression, platelets were activated by incubation with 50µg/mL of CRP-XL at 37°C for 10 mins. After incubation, platelet releasate was collected and used to treat isolated lymphocyte preparations for 4 h at 37°C. Monocytes was then separated and measured for *mPer2* expression (Fig. 4.5). Incubation of monocytes with releasate secreted by activated platelets induced a significant expression of *mPer2* (3.2-fold, $P < 0.01$). This data indicates that the release of soluble mediators upon platelet activation is sufficient for the up-regulation of monocyte *mPer2*.

4.2.5 – Role of TGF-β1 secreted by platelets in monocyte *mPer2* induction

The mechanism which soluble mediators released by platelets conduct to increase monocyte *mPer2* expression was investigated using inhibitors to target specific pathways. Releasates were incubated with neutralising antibodies to TGF-β1 and IL-1β. The platelet releasate was then subsequently added to monocytes for 4 h at 37°C (Fig. 4.5). Antibody directed against TGF-β1 was utilised because TGF-β1 was found to be an important mediator for the induction of monocyte PAI-1, hence it may play a role in the induction monocyte *mPer2* expression. Blocking mAb to IL-1β did not affect *mPer2* levels. In contrast, incubation of monocytes with blocking mAb to TGF-β1 significantly attenuated *mPer2* expression by ~45% ($P < 0.01$).

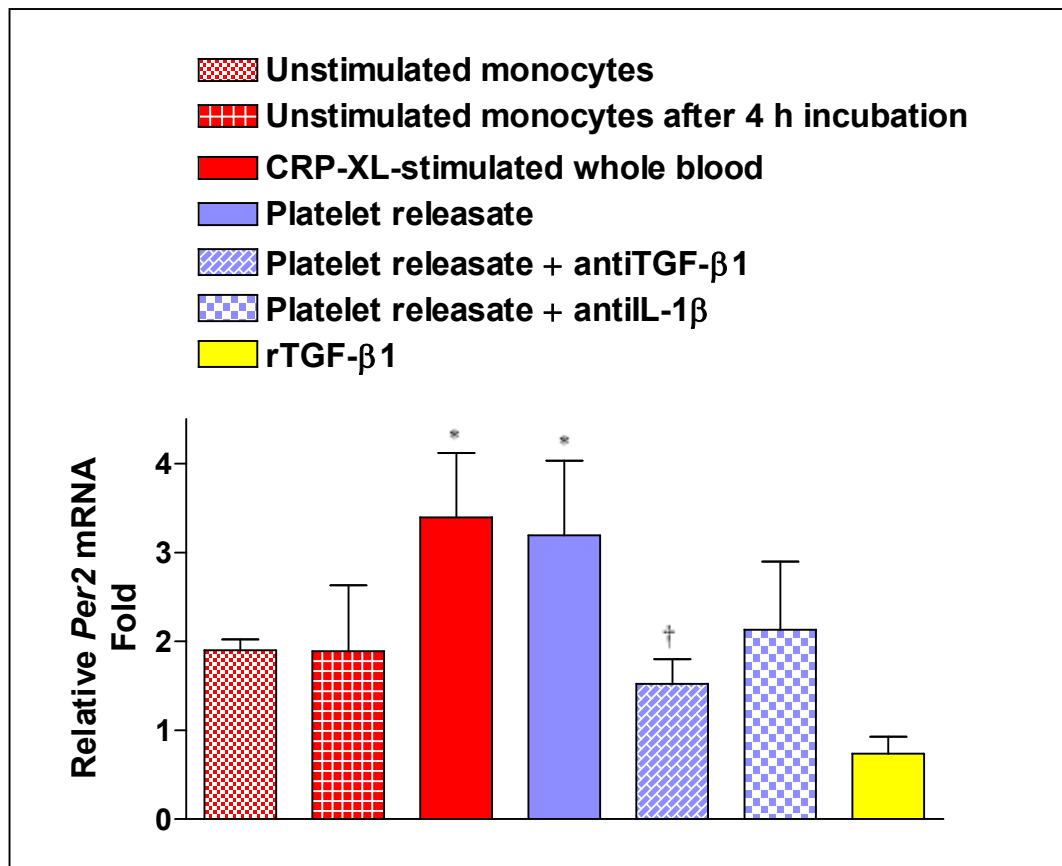


Figure 4.5 – Soluble mediators released by activated platelets induce monocyte *mper2* expression, partially mediated by TGF-β1 (n=6).

Isolated monocytes were incubated with platelet releasates stimulated with CRP-XL. Platelet releasate was sufficient to induce monocyte *mPer2* expression. Neutralising antibodies to IL-1β and TGF-β1 were incubated with platelets releasates. Monocytes were then exposed to this pre-treated platelets releasates. Antibody to TGF-β1 attenuated monocyte *mPer2* expression. *mPer2* mRNA expression determined by real time PCR Values were normalised with *18s* and expressed as mean ± SEM. **P* < 0.05 vs. unstimulated monocytes (control) and †*P* < 0.05 vs. platelet releasate. One-way ANOVA was used for statistical analysis.

These results suggest TGF- β 1 was required for platelet-induced monocyte *mPer2* expression, however other mechanisms are also involved in the regulation.

To demonstrate the hypothesis monocytes were exposed to rTGF- β 1 for 4 h at 37°C. It was observed that rTGF- β 1 alone was not sufficient to induce monocyte *mPer2* expression. The data so far suggests that although TGF- β 1 alone is not sufficient for the induction of monocyte *mPer2* expression, it may play a role with other signalling pathways to regulate *mPer2* expression.

4.3 - Discussion

4.3.1 – The regulation of circadian clock in monocytes

Previous studies have demonstrated that a circadian clock exists in leukocytes, showing diurnal variation in the core clock genes. However, it remains to be elucidated whether a peripheral clock exist in monocytes, and how it is influenced by inflammatory and thrombotic stimuli. In the present study, first evidence is provided that clock gene expression exists in human monocytes. *Dec1* and *Bmal2* expression was regulated in monocytes treated with LPS, while *mPer2* and *Clock* expression was down-regulated. It is unknown whether these clock genes exhibit circadian oscillations in monocytes, and hence are regulated by the circadian clock. Previous reports have demonstrated the circadian expression of *mPer2*, *Bmal1* and *Dec1* in human peripheral blood cells (Boivin *et al.* 2003; Teboul, *et al.* 2005; Fukuya *et al.* 2007) but it is unclear whether *Clock* is expressed in a circadian manner. Takata *et al.* (2002) reported *Clock* mRNA

exhibited no daily variation, while Takimoto *et al.* (2005) showing peak *Clock* expression around midnight. Nevertheless, these reports indicate that clock genes in human peripheral blood cells do exhibit at least some degree of circadian expression.

The response shown by monocyte to LPS suggests that inflammatory stimuli may override the circadian clock mechanism. This hypothesis is supported by a recent study (Okada *et al.* 2008), showing LPS injection significantly suppressed the expression levels of clock genes in both the SCN and liver of rats. Specifically, these authors found that LPS produced early transient suppression of *rPer1* and *rPer2* in the liver, and *rPer2* in SCN, and normalisation of the expression levels was noted the next day. Interestingly, data from Murphy *et al.* (2007) has demonstrated that the administration of LPS results in significant up-regulation of the core clock genes *mPer2* and *Bmal1* in equine blood. However, the authors could not replicate these results when equine peripheral blood mononuclear cells were treated with LPS. These results suggest that there are other mechanisms, or cell types, involved in the regulation of the monocyte circadian clock, and one possibility is platelets. The data in the present study found CRP-XL activated platelets induced monocyte *mPer2* expression, when incubated in whole blood. Previous studies have demonstrated that the synchronization of the circadian clock in the SCN is regulated partially by *mPer2* expression (Challet *et al.* 2003). Assuming that peripheral clocks function in the same manner, this raises the possibility that platelets, following activation, can synchronize the circadian clock in monocytes. Furthermore, the regulation of monocyte *mPer2* expression appeared not to require cell to cell contact, evident in the up-regulation of *mPer2* expression when isolated monocytes were exposed to releasates of activated platelets. Similar to the regulation of PAI-1 in the

previous section, platelets appear to positively regulate *mPer2* by a paracrine mechanism. In contrast to PAI-1, neutralising antibody to TGF- β 1 only partially inhibited platelet-induced *Per-2* expression by ~45% ($P < 0.01$), together with the finding that rTGF- β 1 was not sufficient to increase *Per-2* expression, indicates activated platelets must present one of several signals that regulate monocyte *mPer2*.

In the previous section, the expression of PAI-1 by monocytes was found to be predominantly through stimulation by activated platelets (mainly mediated by TGF- β 1) as opposed to LPS. TGF- β 1 acts through Smad binding element (SBE), three of which exist in the PAI-1 promoter. Hua *et al.* (1999) demonstrated that only two of the three SBE, and more importantly the second E-box (located -566 to -559) are essential for TGF- β -induced promoter activity. Furthermore, work carried out in this laboratory showed the same E-box was important in BMAL2/CLOCK-induced transcription, with 90% of the BMAL2/CLOCK activation abolished when this E-box was mutated (Chong *et al.* 2006). As a result the hypothesis that interaction may occur between clock genes and inflammation/thrombotic stimulus in regulating *Pai-1* expression has arisen. The current data in this section suggests that circadian factors do not play a role in the induction of monocyte PAI-1 by activated platelets. Most of the core clock genes were found to be either unaffected or, in the case of *Clock*, down-regulated when monocytes were stimulated by CRP-XL activated platelets. *mPer2* is involved in the negative loop within the circadian system, and we and others (Schoenhard *et al.* 2003; Chong *et al.* 2006) have shown mPER2 to inhibit activation of *Pai-1* expression by clock factors, hence the up-regulation of *mPer2* is not directly involved in the acute induction of monocyte *Pai-1*. However, the circadian clock cannot fully be discounted in the acute

induction of monocyte PAI-1 until the same stimulation experiments have been carried out at different times of day.

4.4 – Conclusion

The important finding in the present section was that monocytes do express genes of the peripheral clock, which are modulated in response to inflammatory and thrombotic stimuli. Platelets play important roles in both of these pathways. In thrombosis, platelets adhere to the exposed subendothelium following injury to the vessel wall. After tethering, rapid conversion to stable platelet adhesion is required for thrombus formation. During inflammation, platelets mediate capture of monocytes and neutrophils through P-selectin. A key molecular mechanism which platelets participate in and is common to both pathways is the recruitment and interaction of leukocytes into the thrombus. The regulation of several genes has been studied as a result of this interaction including PAI-1 in the previous section. This study has found that platelets have the potential to synchronise the circadian clock in monocytes. The significance of this finding in respect to thrombus formation and inflammation remains to be elucidated, however, the data raises the possibility that platelets may be the link between the circadian clock, thrombosis and inflammation.

The previous section has shown that *Pai-1* mRNA in leukocytes displays diurnal variation under basal conditions which is dependent on the 4G/5G polymorphism. In contrast, the data in the current section suggests that the acute induction of monocyte PAI-1 by either inflammatory or thrombotic stimuli involves a more complicated

mechanism. Under these conditions, the induction of both monocyte *Pai-1* transcript and PAI-1 antigen is independent of the circadian clock.

4.5 – Future work

To explore and validate the studies carried out in this section, a number of investigations could be carried out.

(i) The study of monocyte peripheral clock over time

As the expression of clock genes in monocytes, in the current section, was only examined at one time point, to investigate circadian variation in clock genes expression of monocytes, whole blood could be extracted at different times of the day, with the isolation of monocyte RNA. Various factors such as diet have been reported to affect circadian expression, and therefore subjects would need to be maintained in a consistent environment during experimentation including sleep and diet.

(ii) Increase number of clock genes examined

Besides elevating the time points at which clock gene expression can be examined, the number of clock genes investigated could also be increased. The present study examined 5 clock genes; *Bmal2*, *Dec1*, *Bmal1*, *mPer2*, and *Clock*. Other clock genes that could also be examined include period genes (*Per1* and *Per3*), cryptochrome genes (*Cry1* and *Cry2*) and the nuclear receptor reverse strand c-ERB α (*Rev-erba*). Microarray

methods may be utilised to study the expression of all the clock genes mentioned so far. In addition, it would also give the opportunity to examine other genes that display circadian oscillations, and hence may be regulated by the peripheral circadian clock.

(iii) PGE-2 as a putative signalling pathway to regulate monocyte *mPer2* expression

The present study found that activated platelets up-regulated monocyte *mPer2* expression, while TGF- β 1 partially mediated this response. A putative signalling molecule responsible for the induction of monocyte *mPer2* expression by activated platelets is Prostaglandin E2 (PGE-2). Platelets contain cyclo-oxygenase-1 (COX-1) and it functions to convert arachidonic acid into prostaglandin H2, which in turn is converted to PGE-2. The hypothesis that PGE-2 may act as a monocyte clock resetting agent is supported by previous reports demonstrating PGE-2 as a synchronizer of clock gene expression in a mouse (Tsuchiya *et al.* 2005) and equine model (Murphy *et al.* 2007). To examine this hypothesis, specific inhibitors could be used to suppress COX activity (such as non-steroidal anti-inflammatory drugs, for example aspirin and ibuprofen), and therefore the conversion to PGE-2 in monocyte stimulated with CRP-XL. Additionally, purified PGE-2 may be incubated with monocytes to investigate whether PEG-2 could synchronise the monocyte clock.

(iv) Transfection of clock factors with PAI-1 promoter

This study has not shown whether the expression for *Pai-1* mRNA is directly regulated by the circadian clock. Previous studies have demonstrated *clock* deletion blunted circadian expression of *Pai-1* in the heart, lung and liver in mice (Oishi *et al.* 2006). As transgenic humans are not possible, *in vitro* experiments could be setup. Clock expression plasmids such as BMAL1, BMAL2 and CLOCK could be co-transfected with PAI-1 promoter constructs in monocyte cell lines. This would suggest whether clock factors regulate PAI-1 in monocytes.

Chapter 5

Regulation of platelet *Pai-1* mRNA and protein expression

5.1 – Introduction

The incubation of whole blood with CRP-XL dramatically increased both *Pai-1* mRNA transcript and antigen levels compared to LPS stimulation (section 3), highlighting the importance of platelets in the regulation of monocyte PAI-1 expression. Platelets could also contribute to the elevated PAI-1 antigen levels released into the plasma following CRP-XL activation in whole blood. Platelets can increase PAI-1 levels in the local vicinity by two different mechanisms, firstly high levels of PAI-1 resides in their α -granules, which can be released upon activation. Secondly, platelets contain mRNA which can be used to synthesise new proteins. Previously Brogren *et al.* (2004) have reported that platelets contain mRNA for *Pai-1* and that they can synthesise active PAI-1. Another important finding in the present study was that the appearance of PAI-1 in CRP-XL stimulation of whole blood varied according to the 4G/5G polymorphism. If platelets influence the increased level of PAI-1 antigen in stimulated whole blood, the release or production of PAI-1 by platelets may also be associated with the 4G/5G polymorphism. Linked to the polymorphism is the observation that *Pai-1* mRNA in whole blood under basal conditions exhibited diurnal variation, suggesting that *Pai-1* mRNA abundance may also display diurnal differences in platelets. This part of the thesis therefore examined the possible relationship between the 4G/5G polymorphism, circadian clock, *Pai-1* transcript and protein levels of platelets in resting and activated conditions.

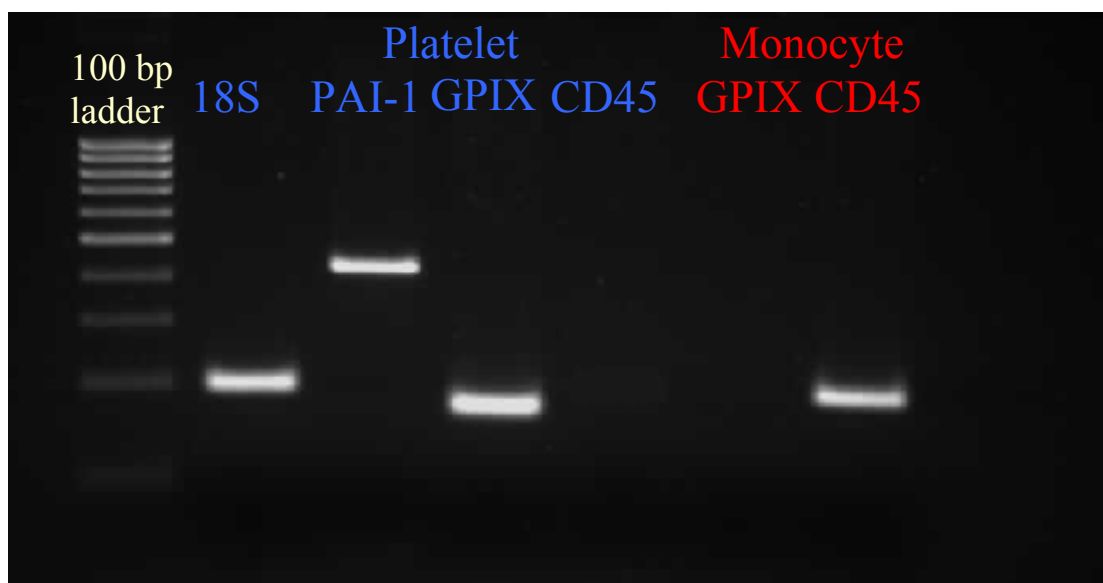


Figure 5.1 – Very low level of leukocyte contamination in the platelet preparations
Semi-quantitative PCR was performed on leukocyte specific marker CD45 on both platelet and monocyte preparations. A very low level of CD45 expression was seen in the platelet preparations, and a high CD45 expression levels in monocyte preparations. In contrast, high expression levels of platelet marker GPIX was detected in platelets isolation, and undetectable in monocyte preparations. Examples 18S and PAI-1 amplicons produced from isolated platelets can also be seen in the figure.

5.2 – Results

5.2.1 – Demonstration that platelets could be isolated free from leukocyte contamination

Platelets were isolated from whole blood, and to ensure that there were no leukocyte contamination in the platelet preparations, semi- quantitative-PCR was performed for the leukocyte specific marker CD45 on cDNA produced from negatively isolated platelets. As shown in figure 5.1, a very weak expression of CD45 could be detected in the platelet preparations, whereas a bright band was observed in the control monocyte preparations. In the figure, expression levels of the platelet-specific marker, glycoprotein-IX (GP-IX) were also measured to demonstrate that the isolated platelet preparations were indeed of platelet origin. Strong expression of GP-IX was measured in isolated platelets, but no detectable signal in the monocyte preparations. These results suggest the isolated platelet preparations contained a very low level of leukocyte contamination.

5.2.2 – Diurnal variation in platelet count but not *Pai-1* mRNA expression

To investigate whether platelet *Pai-1* is regulated by the circadian clock, whole blood was taken from 10 healthy volunteer ($4G/4G = 5$, $4G/5G = 5$) at 9 am (AM) and 4 pm (PM). The time points were chosen in accordance to previously published data on the diurnal rhythms of PAI-1 levels found in human plasma.

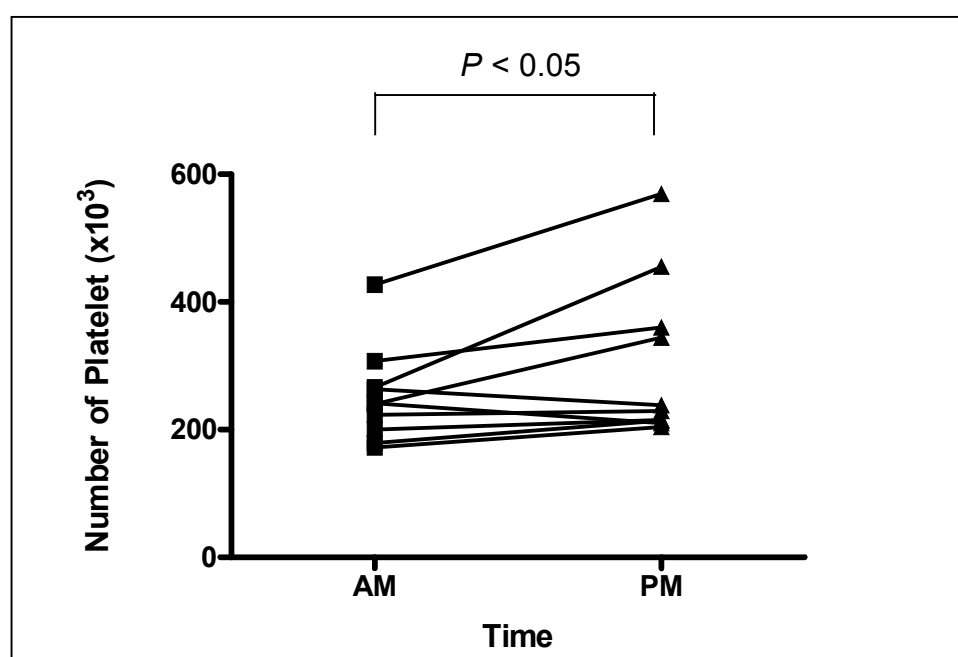


Figure 5.2 – Platelet number exhibit a diurnal variation (n=10)

Platelets were isolated from whole blood taken from 10 subjects at 9 am (AM) and 4 pm (PM). Number of platelets displayed diurnal variation $P < 0.05$. One-way ANOVA was used for statistical analysis.

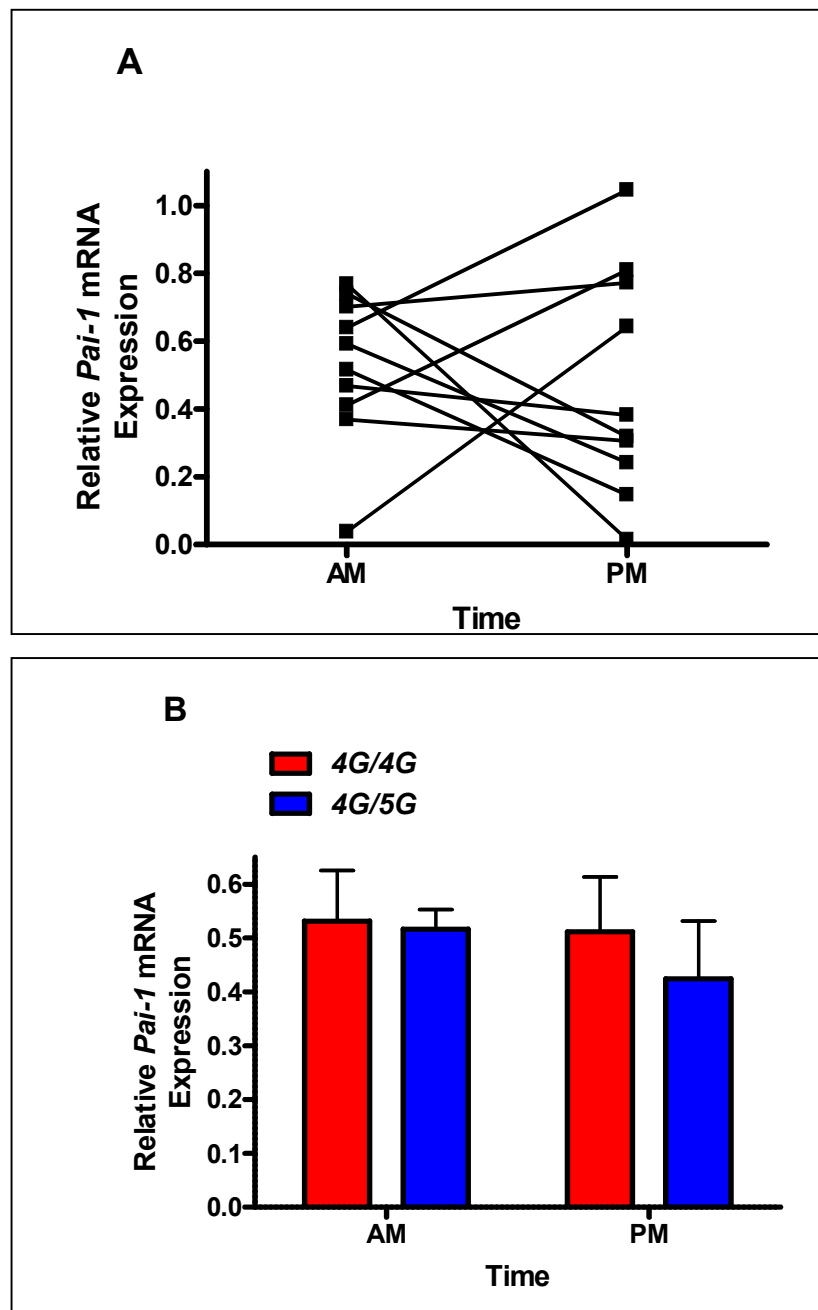


Figure 5.3 – Regulation of platelet *Pai-1* mRNA is independent of circadian clock and polymorphism (n=10, 4G/4G = 5, 4G/5G = 5)

Platelets were isolated from whole blood taken from 10 subjects (5 of each genotype) at 9 am (AM) and 4 pm (PM). (A) Variation in *Pai-1* expression between morning and evening time points are shown for each subject, with values expressed as mean \pm SEM. (B) Time and genotype had no significant influence on the expression of platelet *Pai-1* expression. *Pai-1* mRNA expression determined by real time PCR. Values were normalised with *18s* and shown as mean \pm SEM. Two-way ANOVA was used for statistical analysis.

Platelets were counted and the number of platelets was observed to vary between the two different time points ($P < 0.05$, Fig. 5.2), with the highest average count of platelets being at 4 pm ($304 \pm 40 \times 10^3$ cells/ml) and least at 9 am ($252 \pm 23 \times 10^3$ cells/ml). *Pai-1* mRNA expression was not affected by either time ($F_{(1,18)} = 0.33$, *ns*) or genotype ($F_{(1,18)} = 0.40$, *ns*). Therefore it appears relative *Pai-1* transcript abundance in platelets is not associated with the diurnal variation in platelet count, and is independent of both the circadian clock and the 4G/5G polymorphism.

5.2.3 – Release of PAI-1 by activated platelets

To investigate the possible association between the 4G/5G polymorphism and the level of PAI-1 that could be released by activated platelets, platelets were extracted from 8 volunteers (4G/4G = 4 and 4G/5G = 4). The isolated platelets were counted and normalised to 150×10^3 / μ l for each subject. The extracted platelets were incubated with or without the agonists adenosine diphosphate (ADP), CRP-XL, and thrombin receptor-activating peptide (TRAP) at 37°C. The concentration of each agonist used had been shown by others in the lab to maximally stimulate platelets as measured by flow cytometry. At 10 min, 1 h, 2 h and 4 h, PAI-1 levels were measured by ELISA. PAI-1 antigen was significantly released at all time points by platelets activated by the agonists (Agonist $F_{(3,72)} = 44.03$, $P < 0.0001$; Time $F_{(4,72)} = 131.09$, $P < 0.0001$; T x A interactions $F_{(12,72)} = 17.39$, $P < 0.0001$, showing that the type of agonists and time points interact in their effect on PAI-1 release from platelets). Most of the PAI-1 was released by platelets after 10 min incubation with all agonist tested (Fig. 5.4). TRAP released the most PAI-1 ($15.0 \text{ ng/ml} \pm 1.5$) followed by CRP-XL ($12.2 \text{ ng/ml} \pm 1.0$), with

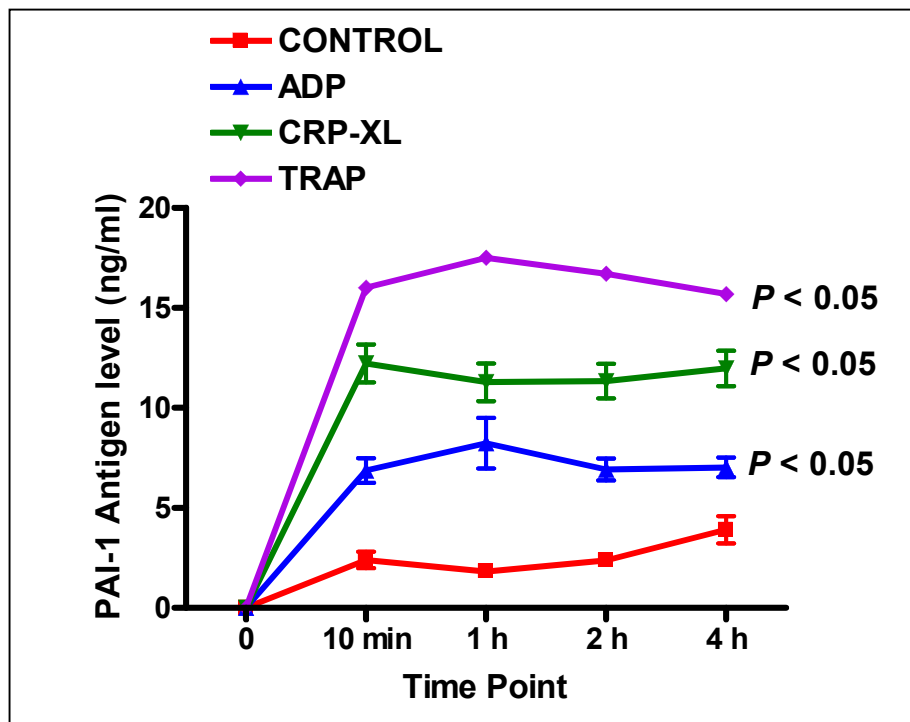


Figure 5.4 – Differential level of PAI-1 released from platelets activated by various agonists (n=7).

Platelets were isolated from whole blood taken from 7 subjects, and incubated with ADP (blue), CRP-XL (green), TRAP (purple), or without (red). At all time points, activated platelets released significantly higher levels of PAI-1 compared to platelets in basal condition (control). Platelets stimulated by TRAP released the most PAI-1, and ADP stimulated platelets released the least. PAI-1 antigen was determined by ELISA. Data expressed as mean \pm SEM. * $P < 0.05$ vs platelets in resting conditions (control). Two-way ANOVA was used for statistical analysis.

ADP resulting in the least (6.9 ng/ml±0.6). Further incubation of agonists with platelets beyond 10 min did not produce significant increase in PAI-1 antigen levels. A slow release of PAI-1 was seen in unstimulated platelets (2.4 ng/ml±0.4). The results so far indicate that synthesis of PAI-1 by activated platelets may not contribute significantly to the level of PAI-1 that can be released.

Platelets originating from donors of *4G* homozygous genotype released significantly higher levels of PAI-1 compared to platelets from heterozygous subjects, following activation with TRAP ($F_{(3,18)} = 7.24$, $P = 0.0359$) CRP-XL ($F_{(1,15)} = 10.80$, $P = 0.0218$), independent of time (Fig. 5.5). The level of PAI-1 released in the *4G* homozygotes was on average 45% higher than in heterozygotes following activation with CRP-XL (15.3 ng/ml±0.8 versus 10.5 ng/ml±0.9, respectively), and 38% under stimulation of TRAP (18.1 ng/ml±1.3 versus 13.1 ng/ml±1.9, respectively). There was no significant of genotype on the level of PAI-1 released by platelets stimulated with ADP. It is clear from the data presented that the release of PAI-1 by activated platelets is regulated by the *4G/5G* polymorphism.

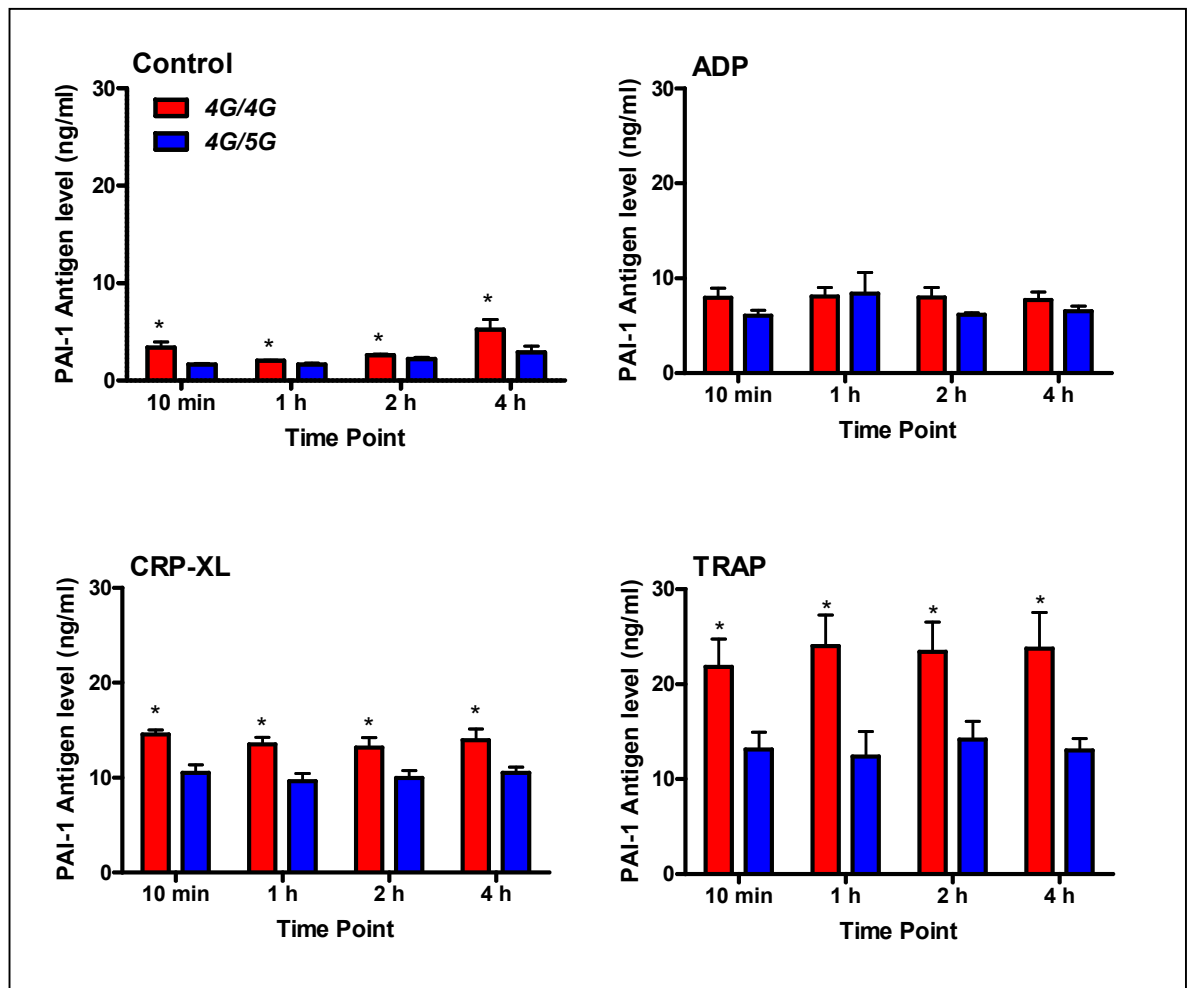


Figure 5.5 – Released of PAI-1 by activated platelets is dependent on the promoter polymorphism (n=7, 4G/4G = 4, 4G/5G = 3).

Platelets were isolated from whole blood taken from 7 subjects, and incubated with ADP, CRP-XL, TRAP, or without. PAI-1 released upon platelet activation by the different agonist was regulated by the 4G/5G polymorphism besides ADP. PAI-1 antigen was determined by ELISA. * $P < 0.05$ vs 4G/5G heterozygous subjects. Data expressed as mean \pm SEM. Two-way ANOVA was used for statistical analysis.

5.2.4 – PAI-1 synthesis by activated platelets

To see if the expression of *Pai-1* was up-regulated in platelets following stimulation, whole blood was taken from 6 healthy donors, and platelets were negatively isolated from PRP. The isolated platelets were counted and normalised to $150 \times 10^3 / \mu\text{l}$ for each subject. The extracted platelets were incubated with TRAP, with or without cycloheximide, or were left untreated at 37°C and *Pai-1* mRNA expression was determined by real-time PCR at 0.5, 1 and 4 h. The mRNA level for *Pai-1* was calculated relative to the 18S housekeeping gene from the same cDNA. The concentration of TRAP used was 1×10^{-3} M, which has been shown by others in the laboratory to maximally stimulate platelets as measured by monocyte platelet aggregate formation and P-selectin expression. The protein synthesis inhibitor cycloheximide was used at $100 \mu\text{g/ml}$ to potentially suppress PAI-1 synthesis.

Significant Time effect ($F_{(3,36)} = 6.92$, $P = 0.0009$) was detected but treatment with TRAP or cycloheximide were insignificant ($F_{(5,36)} = 0.29$, *ns*) on the relative abundance of *Pai-1* transcript in platelets (Fig. 5.6). However, Bonferroni tests showed that platelets activated by TRAP after 4 h caused a significant reduction in *Pai-1* expression ($P < 0.05$). The lowering of *Pai-1* expression was inhibited in the presence of cycloheximide. The data indicate that activation of platelets results in degradation of *Pai-1* transcript which is potentially used to synthesise new PAI-1 protein.

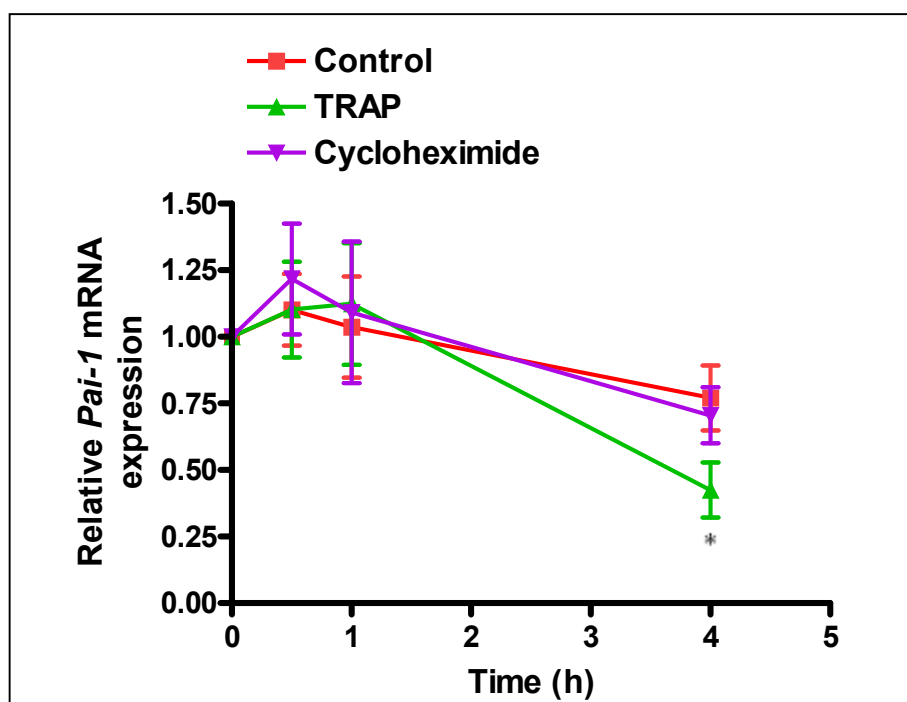


Figure 5.6 – Expression of *Pai-1* mRNA in activated platelets (n=6)

Platelets were isolated from whole blood taken from 6 subjects, and incubated with either TRAP (green) or TRAP and cycloheximide (purple), or without (red). Activation of platelets by TRAP produced rapid *Pai-1* mRNA degradation. *Pai-1* mRNA expression determined by real time PCR. Values were normalised to *18s*. * $P < 0.05$ vs unstimulated platelets and in the presence of cycloheximide. Data expressed as mean \pm SEM. Two-way ANOVA was used for statistical analysis.

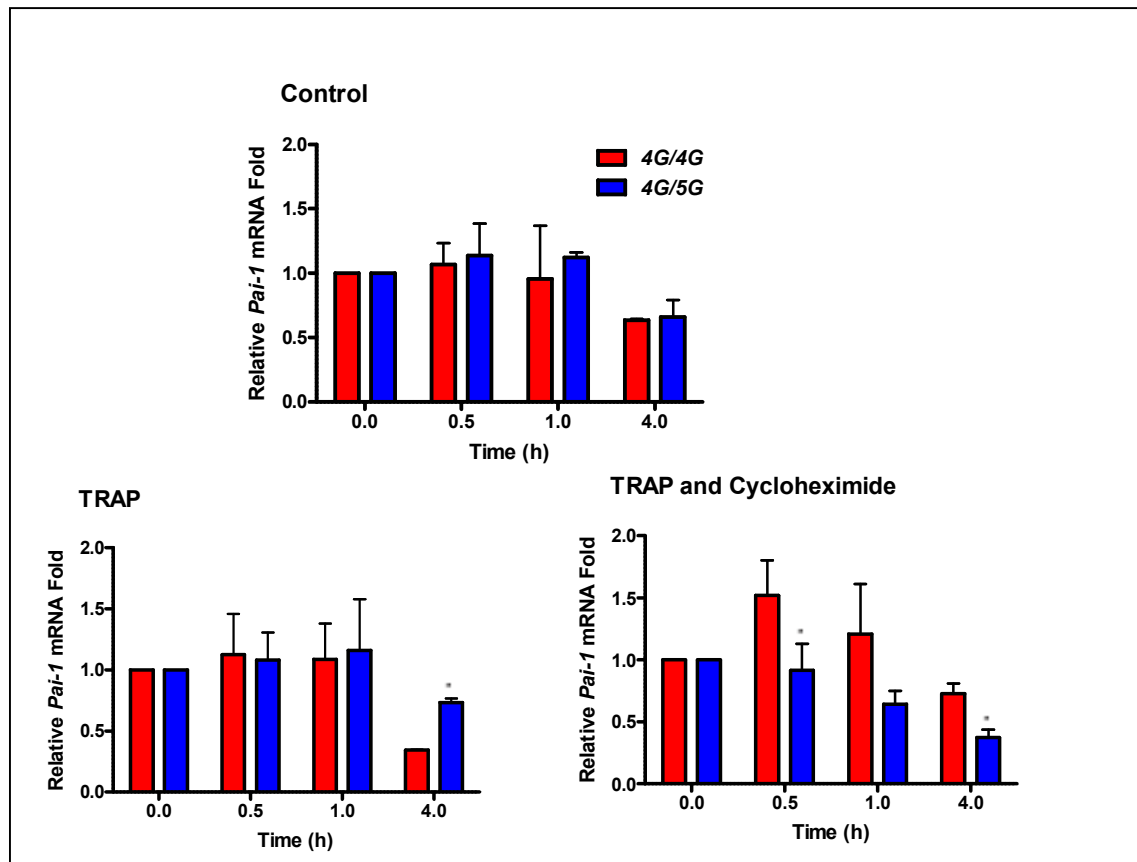


Figure 5.7 – Effect of genotype on *Pai-1* mRNA expression in activated platelets (n=6, 4G/4G = 3, 4G/5G = 3).

Platelets were isolated from whole blood taken from 6 subjects, and incubated without any agonist (A), or with either TRAP (B) or TRAP and cycloheximide (C). Degradation of *Pai-1* transcript was faster in activated platelets from 4G homozygous subjects than heterozygous subjects $P < 0.05$. *Pai-1* mRNA expression determined by real time PCR. Values were normalised with 18s. * $P < 0.05$ vs 4G/4G homozygous subjects. All data expressed as mean \pm SEM. Two-way ANOVA was used for statistical analysis.

No effect of genotype on *Pai-1* mRNA abundance was observed in platelets under basal conditions (Genotype $F_{(1,12)} = 0.34$, *ns*; Time $F_{(3,12)} = 2.16$, *ns*). This was also true for platelets stimulated with TRAP (Genotype $F_{(1,12)} = 0.31$, *ns*; Time $F_{(3,12)} = 3.22$, $P = ns$). However, significant effect of genotype was observed when cycloheximide was incubated along with TRAP on platelet *Pai-1* expression (Genotype $F_{(1,12)} = 13.86$, $P = 0.0204$; Time $F_{(3,12)} = 3.45$, $P = 0.0515$). Furthermore, the rate of degradation in *Pai-1* mRNA was 50% faster in platelets from 4G homozygotes compared to heterozygotes ($P < 0.05$) after 4 h incubation with TRAP (0.7 ± 0.03 versus 0.5 ± 0.13). This genotype difference was reversed with the co-incubation of cycloheximide, where *Pai-1* transcript in platelets isolated from 4G homozygotes degraded 65% slower than *Pai-1* mRNA in platelets from heterozygous origin ($P < 0.05$; 0.4 ± 0.06 versus 0.7 ± 0.08). The data so far suggest platelets from 4G homozygous genotype may be able to synthesise more PAI-1 than platelets of heterozygous origin. Furthermore the polymorphism may exert its effect through the stabilisation of the *Pai-1* transcript.

It was observed from section 4.2.3 that activation of platelets resulted in the release of pre-formed PAI-1 from their α -granules. To measure PAI-1 antigen in the platelet cytoplasm extracted platelets were incubated with TRAP, or TRAP and cycloheximide, or were left untreated at 37°C. At time points 0, 0.5, 1 and 4 h, the platelets were lysed, and total PAI-1 antigen was measured. It was evident PAI-1 was acutely released by activated platelets, as a dramatic reduction of PAI-1 protein was observed between 0 and 0.5 h in platelets activated by TRAP with or without the co-incubation of cycloheximide (Fig. 5.8). The activation of platelets with TRAP increased the level of

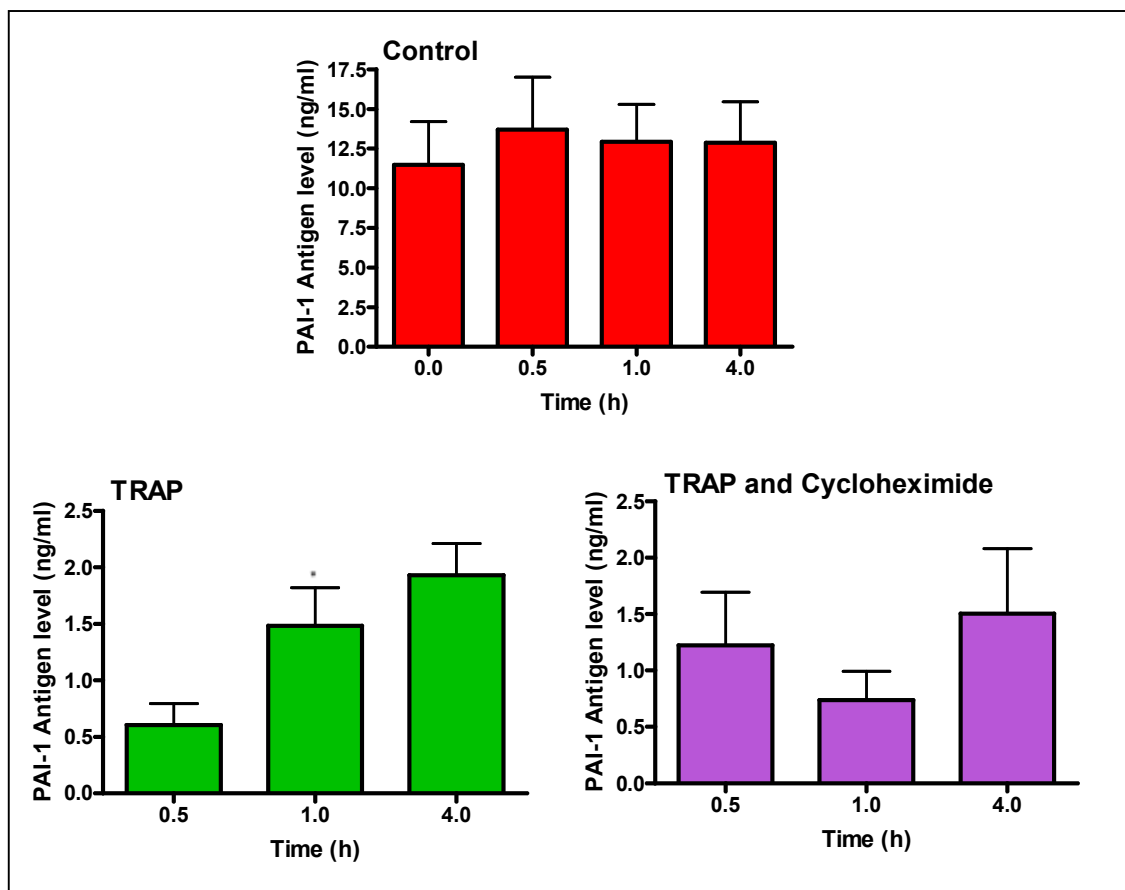


Figure 5.8 – Cycloheximide partially suppressed the production of PAI-1 by activated platelets (n=7).

Platelets were isolated from whole blood taken from 7 subjects, and incubated with either TRAP or TRAP and cycloheximide, or without. Platelets activated by TRAP synthesize PAI-1, inhibited by cycloheximide. PAI-1 antigen was determined by ELISA. * $P < 0.05$ vs 0.5 h. All data expressed as mean \pm SEM. One-way ANOVA was used for statistical analysis.

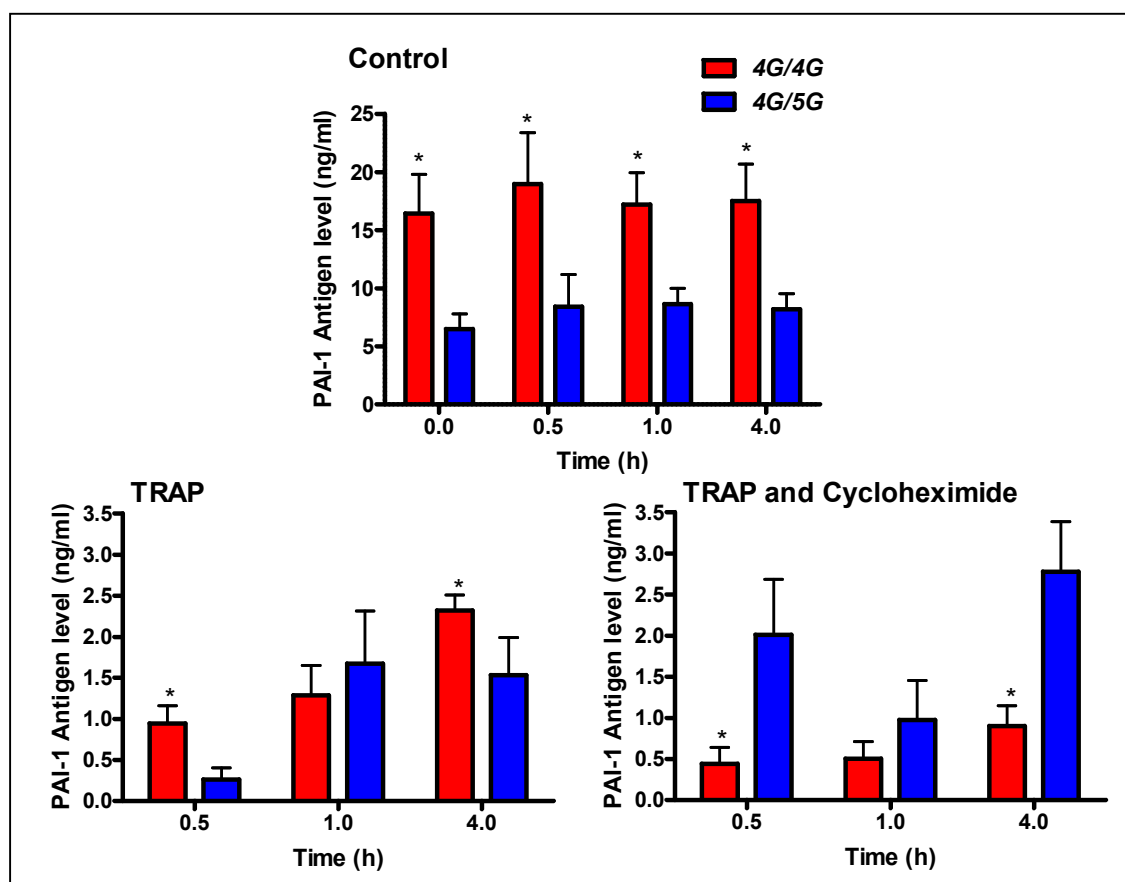


Figure 5.9 – Effect of genotype on PAI-1 production in activated platelets (n=6, 4G/4G = 3, 4G/5G = 3).

Platelets were isolated from whole blood taken from 6 subjects, and incubated without any agonist, or with either or TRAP and cycloheximide. Increased PAI-1 synthesis was observed in activated platelets from 4G homozygous subjects compared to heterozygous subjects. PAI-1 antigen was determined by ELISA. * $P < 0.05$ vs 4G/5G heterozygous subjects. All data expressed as mean \pm SEM. Two-way ANOVA was used for statistical analysis.

PAI-1 in platelets from 0.5-4 h but only significant between 0.5 and 1 h ($0.6 \text{ ng/ml} \pm 0.2$ and $1.5 \text{ ng/ml} \pm 0.3$ respectively). The abundance of PAI-1 in platelets was found not to vary significantly from 0.5-4 h by the addition of cycloheximide. These observations indicate platelets can synthesise PAI-1, which maybe released after a threshold following stimulation.

ANOVA revealed a significant effects in PAI-1 levels as predicted from the results in section 5.2.3 (Genotype $F_{(1,12)} = 10.86$, $P = 0.0301$; Time $F_{(3,12)} = 1.48$, *ns*) resulting in average 47% higher PAI-1 levels in platelets *4G* homozygous subjects compared to heterozygotes (Fig. 5.9). In TRAP stimulated platelets, significant Time effect only was observed ($F_{(2,8)} = 18.43$, $P = 0.001$, Genotype $F_{(1,8)} = 0.60$, *ns*). However, Bonferroni tests indicates platelets from *4G* homozygotes synthesised significantly higher levels of PAI-1 compared to heterozygotes ($P < 0.05$) at 0.5 and 4 h. Significant Time, Genotype and T x G interaction effects on platelet PAI-1 synthesis were also observed (Time $F_{(2,8)} = 12.09$, $P = 0.0038$, genotype $F_{(1,8)} = 5.12$, $P = 0.00864$, T x G $F_{(2,8)} = 5.43$, $P = 0.0323$). Consistent with data on *Pai-1* transcript abundance, the co-incubation of cycloheximide with TRAP resulted in 39% increase of PAI-1 protein in platelets originating from heterozygous genotype when compared to *4G* homozygous genotype.

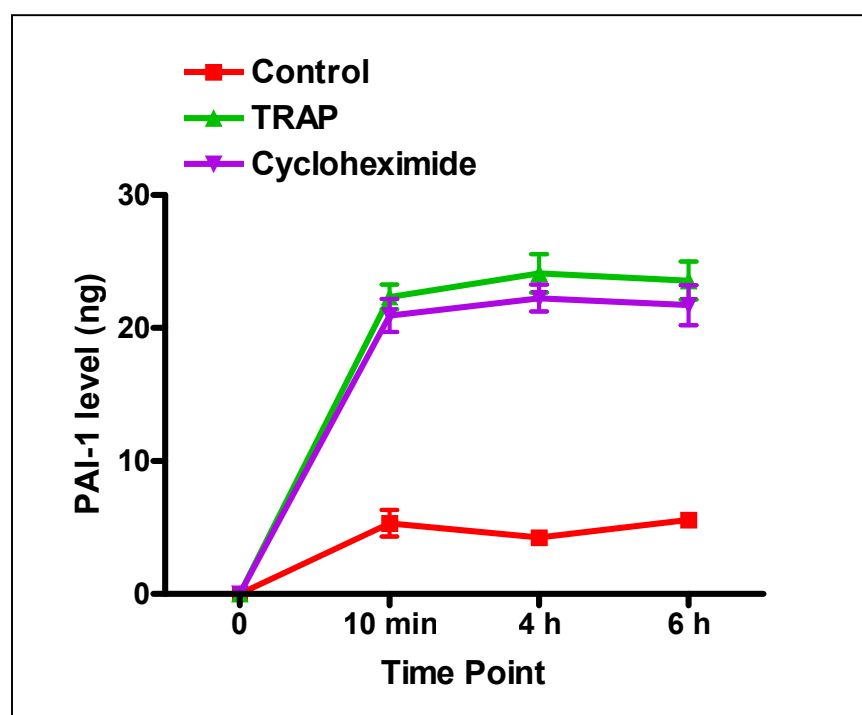


Figure 5.10 – Cycloheximide partially suppressed the production of PAI-1 by activated platelets (n=7).

Platelets were isolated from whole blood taken from 7 subjects, and incubated with either TRAP (green) or TRAP and cycloheximide (purple), or without (red). Production of PAI-1 by platelets activated by TRAP was partially inhibited by cycloheximide. PAI-1 antigen was determined by ELISA. Data expressed as mean \pm SEM. Two-way ANOVA was used for statistical analysis.

The data presented supports the idea that the synthesis of PAI-1 in platelets is dependent on the *4G/5G* polymorphism.

To investigate the contribution of synthesised PAI-1 on the level of PAI-1 produced by activated platelets, isolated platelets were incubated with TRAP, or TRAP and cycloheximide, or were left untreated at 37°C and total amount of PAI-1 antigen was analysed by ELISA at 10 min, 4 h and 6 h. Most of the PAI-1 was released by platelets after 10 min of incubation with TRAP, as shown in the previous section 4.2.3 (Fig. 5.10). The co-incubation of TRAP with cycloheximide resulted in an insignificant suppression of PAI-1 in activated platelets. The average inhibition caused by cycloheximide was only 10% (22.3 ng±0.9 with TRAP versus 20.9 ng±1.24 with cycloheximide), hence casting doubt on the relative contribution of PAI-1 synthesised by activated platelets to the acutely released local level of PAI-1 at the site of injury. However, the delay of PAI-1 synthesis may be a way of stabilising the clot after a few hours.

5.3 – Discussion

5.3.1 – Leukocyte contamination in platelet preparations

The identification of platelet-specific mRNA is often limited by contamination of platelet preparations with leukocytes. Since the amount of mRNA in single leukocytes (approximately 25 fg mRNA/leukocyte) is much higher than in platelets (approximately 0.002 fg mRNA/platelet), a small number of leukocytes can significantly distort the

profile of platelet gene expression. To overcome this problem, the present study negatively isolated platelets by selectively removing leukocyte contamination using specific antibody coated magnetic beads. This technique of leukocyte depletion is a reliable tool for the study of platelet mRNA transcript, as evidenced by the very low expression of the leukocyte marker CD45 in the platelet preparations. Hence, this negative platelet isolation method was used in the present study to examine *Pai-1* mRNA transcript.

5.3.2 – The role of the circadian clock in the regulation of platelet *Pai-1* expression

Previous reports have demonstrated that circulating platelets exhibit a circadian oscillation, with peak platelet count in the late afternoon around 17:00 h (Kanabrocki *et al.* 1999; Bremmer *et al.* 2000). Consistent with these reports, the current study shows a significant increase in platelet count during the afternoon compared to the morning. These results together with a recent report by Ohkura's group (2008) demonstrating the disruption of *clock gene* resulted in the loss of day/night fluctuations in platelet aggregation in mice, lead us to investigate whether platelet *Pai-1* mRNA levels may also display diurnal variation. However, the data presented in this section showed no significant difference in *Pai-1* transcript abundance between morning and afternoon in platelets under basal conditions, and hence the diurnal variation in the amount of circulating platelets does not appear to associate with *Pai-1* mRNA levels. The differences between younger and mature platelets may explain the lack of association in platelet *Pai-1* expression and platelet count. The total platelet count does not differentiate between younger and mature platelets. It would be expected that younger

platelets contain more *Pai-1* mRNA than mature platelets, as some of the *Pai-1* mRNA would be degraded/translated in the latter. Furthermore, it has been reported that younger platelets have a higher capability for protein synthesis than mature platelets. Evidence was first provided by Booyse and Rafelson in 1967, where they investigated the protein synthetic activity of various platelet populations, and found the strongest activity detectable in young platelets. Later, Kieffer *et al.* (1987) showed that newly formed platelets isolated from splenectomised patients with idiopathic thrombocytopenic purpura (ITP) incorporated radio-labelled amino-acids at a significantly higher rate than from normal adult donors. Hence a high platelet count may not necessarily produce a higher *Pai-1* expression. Addressing this issue would require a method that distinguishes between young and mature platelets.

Consistent with a recent report by Brogren *et al.* (2008), the present study found a lack of association between *Pai-1* mRNA and genotype, indicating that *Pai-1* expression in platelets under basal condition is independent of both circadian clock and *4G/5G* polymorphism.

5.3.3 – Influence of the *4G/5G* polymorphism on the release of PAI-1 from activated platelets

Large amount of PAI-1 resides in platelet α -granules, and it has been previously demonstrated the activation of platelets results in the acute release of PAI-1. However, it is unknown whether the amount released is dependent upon the *4G/5G* polymorphism. In this study, platelets were stimulated by various agonists and it was observed that the

highest level of PAI-1 was released by platelets activated with TRAP, the least with ADP, and the amount of PAI-1 released by CRP-XL stimulated platelets falls between the other two agonists. This differential platelet activation by a range of agonists has also been observed in other reports; Janes *et al.* (1993) demonstrated ADP stimulation of platelets resulted in partial deregulation, as detected by the expression of P-selectin and CD63, Klinkhardt *et al.* (2003) found that activation was increased in platelets incubated with TRAP compared to ADP stimulation by measuring the expression of CD62p and the formation of platelet-leukocyte aggregates. The level of PAI-1 released by stimulated platelets appears to be associated with the level of degranulation caused by the activation of platelets.

The present study shows an association between the *4G/5G* promoter polymorphism and PAI-1 released from activated platelets. There are two potential mechanisms platelets can acquire PAI-1 that could account for the genotype difference; endogenous synthesis by megakaryocytes and endocytosis of exogenous PAI-1 in plasma. Previous studies have demonstrated that *4G* homozygous individuals display highest PAI-1 plasma levels compared to donors of the other two genotype (Margaglione *et al.* 1998). Taken together with the results in the current section suggests platelets from *4G* homozygous genotype individuals released higher level of PAI-1 than heterozygous subjects, indicate platelets obtain PAI-1 mainly by endocytosis of plasma PAI-1. Platelet secretory proteins that originate from the plasma have similar concentrations to that seen in plasma, as shown for the *in vivo* uptake IgG and fibrinogen into the α -granules of human platelets (Harrison *et al.* 1989).

The correlation between PAI-1 content and plasma levels (measured in chapter 3) was determined in the present study, and it was observed that the average platelet concentration (average 20 ng/ml) was far higher than that of plasma concentration (average 10 ng/ml), which is consistent with the study by Brogren group (2008). These observations suggest that PAI-1 is mainly acquired through endogenous synthesis either in the megakaryocyte or the platelet, since α -Granule proteins that are synthesised by megakaryocytes have greater concentrations in platelets relative to plasma, as demonstrated for β -thromboglobulin, platelet factor 4 (Ryo *et al.* 1983; Sottile *et al.* 1989), von Willebrand factor and coagulation factor V (Sporn *et al.* 1985; Chiu *et al.* 1985). Studies carried out in cell lines by two different groups further suggest that PAI-1 in platelet α -granules originate from the production by megakaryocytes. Chuang and Schleef (2001) using the megakaryocytic cell line MEG-01 demonstrated Semliki Forest virus (SFV) transfer mediated gene expression enhanced PAI-1 storage by 6-9 fold; furthermore particles from SFV/PAI-1 infected cells displayed a 5-fold enhanced secretion of PAI-1 following treatment with ADP compared to particles incubated in the absence of secretagogue. Results from Madoiwa *et al.* (1999) indicated that both PAI-1 mRNA and *de novo* synthesis of PAI-1 protein is induced after differentiation of immature progenitor cells into megakaryocytes. These data were obtained through the use of differentiating megakaryocytic progenitor cells and CD34⁺/CD41⁻ cells from cord blood.

Assuming from the evidence provided that platelet PAI-1 is acquired via endogenous synthesis in megakaryocytes, questions arise regarding the functionality of the promoter 4G/5G polymorphism in megakaryocytes. As platelets from 4G homozygous subjects

released more PAI-1 than heterozygous donors, intuitive speculation leads to the idea that the polymorphism does play a role in the regulation of PAI-1 in megakaryocytes. However, further studies are required to test this hypothesis.

5.3.4 – Influence of the 4G/5G polymorphism on the synthesis of PAI-1 from activated platelets

In addition to the ability of platelets to release PAI-1 on stimulation, activated platelets may also synthesise PAI-1. Activation-dependent synthesis of proteins is contrary to conventional expectations because platelets are anucleate. However, early (Roth *et al.* 1989) and recent (Weyrich *et al.* 1998; Lindeman *et al.* 2001; Reviewed in Harrison and Goodall 2008) observations demonstrate that mature circulating platelets have a significant complement of mRNA generated by precursor megakaryocytes. Furthermore platelets translate some of these constitutive transcripts in response to activating signals (Lindeman *et al.* 2001). Specifically, Brogren *et al.* (2004) reported that platelets contain mRNA for PAI-1 and that there is constitutive *de novo* synthesis of active PAI-1 following platelet stimulation. To confirm platelet synthesis of PAI-1, both the levels of PAI-1 transcript and protein was studied in activated platelets. First, the release of preformed PAI-1 by degranulated platelets were studied, then PAI-1 antigen which may reside in the platelet cytoplasm was examined.

The present study demonstrated that activation by TRAP generated a rapid degradation of *Pai-1* transcript in platelets by 4 h. The translation of *Pai-1* mRNA into PAI-1 protein may accelerate the rate of transcript degradation, suggesting that platelets can

synthesise new PAI-1. Interestingly, the increased rate of *Pai-1* mRNA degradation was attenuated by the protein synthesis blocker cycloheximide. This inhibitor functions by blocking tRNA binding and release from ribosome (Obrig *et al.* 1971). The mechanism involved in the inhibition of transcript degradation is unknown but it may possibly act by interfering with either the initial endonucleolytic cleavage or in the steps involved in the breakdown of the mRNA into fragments. Consistent with the *Pai-1* transcript data, PAI-1 protein levels were increased in platelets following TRAP activation. The ability for platelets to synthesise PAI-1 was further evident when cycloheximide attenuated TRAP-dependent PAI-1 expression. The amount of PAI-1 synthesised in platelets appeared to be relatively minor compared to the level of PAI-1 released. These results together with work carried out by Brogren's group (2004), which showed that the increase in the level of PAI-1 released following thrombin stimulation became blunt after 3 h, indicates that synthesised PAI-1 is likely to be of minor importance to the level of PAI-1 released by activated platelets.

Following 6 h incubation, the rate of *Pai-1* mRNA degradation appeared to be greater in TRAP activated platelets from *4G* homozygous subjects than heterozygous subjects. On the assumption that the accelerated decay rate of *Pai-1* transcript is due to increased PAI-1 protein synthesis, the data indicate that platelets isolated from *4G* homozygous donors would produce higher level of PAI-1 than their heterozygous counterparts. Indeed, TRAP activated platelets from *4G* homozygous subjects appeared to synthesise increased level of PAI-1 compared to platelets from heterozygous donors. Interestingly, the introduction of cycloheximide to TRAP activated platelets was found to reverse this trend. A possible mechanism for this result is that the addition of cycloheximide may

inhibit proteins that affect the stability of *Pai-1* transcript, and cause the reversal of genotype effect on PAI-1 expression through post-transcriptional mechanisms. miRNA has been demonstrated to be expressed in megakaryocytes (Garzon *et al.* 2006), and may possibly be present in platelets. The existence of miRNA in platelets could affect *Pai-1* mRNA stability, and would be a suitable candidate for future investigations.

In the current section, the level of PAI-1 produced by activated platelets was measured in terms of total PAI-1 antigen, which allows for the comparison between the level of PAI-1 mRNA and proteins contained and produced by the platelets. However, the importance of detecting the activity of PAI-1 released/synthesised by activated platelets is also recognised, for the ability to determine the relative contribution platelets make to the resistance of clots to fibrinolytic degradation. The conformation of PAI-1 in platelets is still a debate; early studies have indicated that only 5% to 10% of PAI-1 in platelets is in the active form (Schleef *et al.* 1985; Booth *et al.* 1988), however, other studies have shown that PAI-1 activity could be as high as 60% (Lang and Schleef 1996; Nordenhem *et al.* 1997), and that there is a continuous production of large amounts of active PAI-1 in platelets (Brogren *et al.* 2004). Thus, to confirm that the release of PAI-1 is the mechanism by which platelets contribute to the stabilisation of blood clots, the activity of PAI-1 released by activated platelets must be determined.

5.4 – Conclusion

The present study has demonstrated platelets originating from *4G* homozygotes subjects contained on average 38% more PAI-1 than their heterozygous counterparts which could be released upon degranulation. Furthermore, the activation of platelets through the thrombin PAR1 receptor accelerated the degradation of *Pai-1* transcript in *4G* homozygotes compared to heterozygotes. The relative contribution made by synthesis of PAI-1 to the overall level of PAI-1 in platelets was low. Nevertheless, these observations suggest that the association between the *4G/5G* polymorphism and thrombotic events could be mediated by an effect on the secretion and/or synthesis of PAI-1 by platelets in addition to the plasma levels of the inhibitor.

Circulating level of PAI-1 in human plasma display circadian variation that is linked to the *4G/5G* polymorphism. It has been demonstrated that *Pai-1* mRNA expression in platelets under basal conditions is independent of circadian clock and promoter polymorphism. However, the results showing increased secretion of PAI-1 from platelets of *4G* homozygous subjects suggest that the circadian clock may influence PAI-1 level in platelets through other mechanisms. Previous studies have shown that PAI-1 in the α -granule of platelets may originate from endogenous synthesis in megakaryocytes. Hence, it is possible that time-dependent levels of PAI-1 may be packed inside platelet α -granule during differentiation of mature megakaryocytes.

5.5 – Future Work

To support the hypotheses raised in this section, further experiments needs to be carried out.

(i) Putative influence of 4G/5G polymorphism on regulation of PAI-1 in megakaryocytes

Evidence from the previous studies (Madoiwa *et al.* 1999; Chuang and Schleef 2001) indicates PAI-1 found in α -granule of platelets originates from the endogenous synthesis in megakaryocytes, which is supported by the present study. The present study demonstrated platelets from 4G homozygous subjects released more PAI-1 than heterozygous subjects, suggesting the promoter 4G/5G polymorphism is regulating the level of PAI-1 in megakaryocytes. The regulation of PAI-1 by the polymorphism could be investigated using megakaryocytes from genotyped subjects. Transfection of PAI-1 construct with clock expression plasmid in megakaryocytic cell lines would give the opportunity to examine both the circadian and the polymorphism regulation of *Pai-1* in this cell type. PAI-1 protein levels could be investigated by lysing mature megakaryocytes, and detecting PAI-1 antigen by western blot or ELISA.

(ii) Alternative protein synthesis inhibitor

The present study examined the synthesis of platelet PAI-1 by blocking protein synthesis with cycloheximide. However, only 10% of total PAI-1 antigen secreted by

activated platelets was shown to be inhibited, and communication with other groups (Brogren, Sweden) revealed cycloheximide may not be an appropriate inhibitor. The use of puromycin to suppress protein synthesis could be a potential alternative, which inhibits translation by prematurely terminating peptide chains. Brogren *et al.* 2004, demonstrated addition of puromycin to platelets resulted in the partial inhibition of PAI-1 synthesis. Another inhibitor that could be utilized to suppress PAI-1 synthesis in platelets is rapamycin, which attenuates protein synthesis by blocking the action of mTOR proteins. Recently (Pontrelli *et al.* 2008), rapamycin has been shown to inhibit PAI-1 expression in proximal tubular cells. To further investigate the relative contribution of synthesis of PAI-1 to the total level of PAI-1 produced by platelets, both puromycin and rapamycin appears to be appropriate alternatives to cycloheximide.

(iii) Metabolic radiolabeling and immunoprecipitation as an alternative approach for detecting PAI-1 synthesis

The present study only detected small amount of increase in synthesized PAI-1 by activated platelets, and it is not clear with the use of cycloheximide, as to whether platelets under stimulation synthesises PAI-1. Another method to examine the synthesis of PAI-1 is incorporating metabolic radiolabeling of PAI-1 followed by immunoprecipitation. Synthesis of PAI-1 by activated platelets would be evident in the amount of increasing radio active PAI-1.

(iv) The regulation of PAI-1 expression by platelet aggregation

The level of increase in PAI-1 expression in TRAP-activated platelets was very low in the present study. Synthesis of platelet PAI-1 may be regulated by the aggregation of activated platelets. The current study avoided the formation of platelet aggregates during activation, and therefore the contribution from binding between platelets to the synthesis of PAI-1 was not examined. The hypothesis that platelet aggregation is important in the regulation of PAI-1 synthesis is supported by studies carried out with Bcl3. Weyrich *et al.* (1998) demonstrated B cell lymphoma 3 (Bcl3) expression was enhanced in platelet aggregates in comparison to single cells in suspension. Later the same group (Pabla *et al.* 1999) reported that the engagement of integrin $\alpha\text{IIb}\beta_3$, or an activating antibody to this antigen induced synthesis of Bcl-3 in the absence of thrombin. Furthermore antibodies or peptides that block $\alpha\text{IIb}\beta_3$ engagement and platelet aggregation attenuated Bcl-3 synthesis in response to thrombin. As the principal mechanism for the binding between platelets involves binding of fibrinogen $\alpha\text{IIb}/\beta_3$ integrin, to investigate the influence of aggregation of stimulated platelets on PAI-1 synthesis, adherence of platelets to fibrinogen could be examined. Increases in PAI-1 protein after adherence of platelets to fibrinogen would suggest the importance of aggregation of activated platelets to PAI-1 synthesis.

(v) Activity of platelet PAI-1

The present study detected total PAI-1 antigen released by activated platelets, hence the activity of PAI-1 was not determined. The activity of PAI-1 has implications of how

platelets may contribute the resistance of clots to fibrinolytic lysis. Because the mechanism involved in the stability of PAI-1 (reaction of PAI-1 and tPA) is rather complicated, it is difficult to determine the activity of PAI-1. To investigate the active configuration of PAI-1, a functional assay used in Brogren *et al.* (2004) could be used. The basis of this approach is that only active PAI-1 is capable of forming a stoichiometric 1:1 complex with tPA. By incorporating metabolic radiolabeling of PAI-1 in the presence of tPA, tPA/PAI-1 complex can be detected as a measure of PAI-1 activity.

Chapter 6

Clot formation and lysis

6.1 – Introduction

In this chapter the effect of both the promoter *4G/5G* polymorphism and circadian factors on thrombolysis, was assessed. The rationale for this view stem from previous experiments demonstrating that the *4G/5G* polymorphism regulates the amount of PAI-1 produced by monocytes and platelets. From these data, it can be deduced that activation of platelets and monocytes from a *4G/4G* homozygous individual could result in a higher local PAI-1 concentrations in thrombi than in heterozygous subject, therefore contributing to an increase in thrombolysis resistance. To study the effects of circadian and *4G/5G* polymorphism on the stability of the clot, two systems were employed to create artificial thrombi; the Chandler loop and rotation thromboelastometry (TEM).

6.2 – Results

6.2.1 – Chandler loop thrombi

Whole blood was taken from the same 10 volunteers (five homozygous for *4G* and five heterozygous) at two time points; 9 am and 4 pm. Blood was recalcified with 0.25M CaCl_2 , and mixed with 0.9% saline, and FITC-fibrinogen as described previously (section 2.5.2), and the loop was rotated for 2 hrs at 10 r.p.m. The resulting clot was washed and blotted, and the weight recorded. ANOVA revealed that Time ($F_{(1,8)} = 1.83$, *ns*) and Genotype ($F_{(1,8)} = 1.56$, *ns*) did not appear to have a significant effect on the weight of the thrombi (Fig. 6.1).

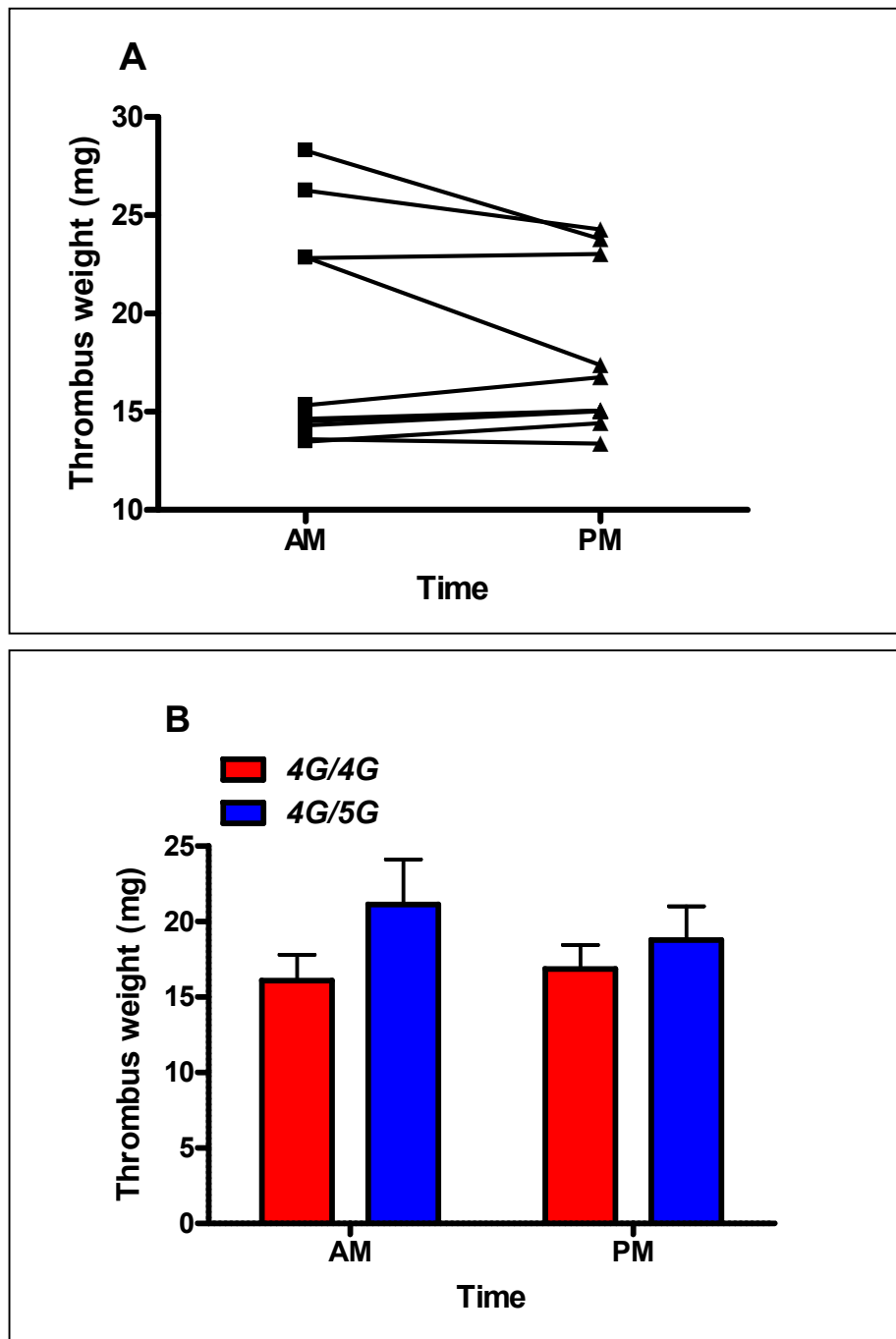


Figure 6.1 –Variation of thrombus weight (n=10, 5 in each genotype).

Thrombi were produced from whole blood of 10 volunteers in the Chandler loop at 9 am (AM) and 4 pm (PM). (A) Variation in thrombus weight between morning and evening time points are shown for each subject. (B) The weight of the thrombus was not significantly affected by time or genotype. All values expressed as mean \pm SEM. Two-way ANOVA was used for statistical analysis.

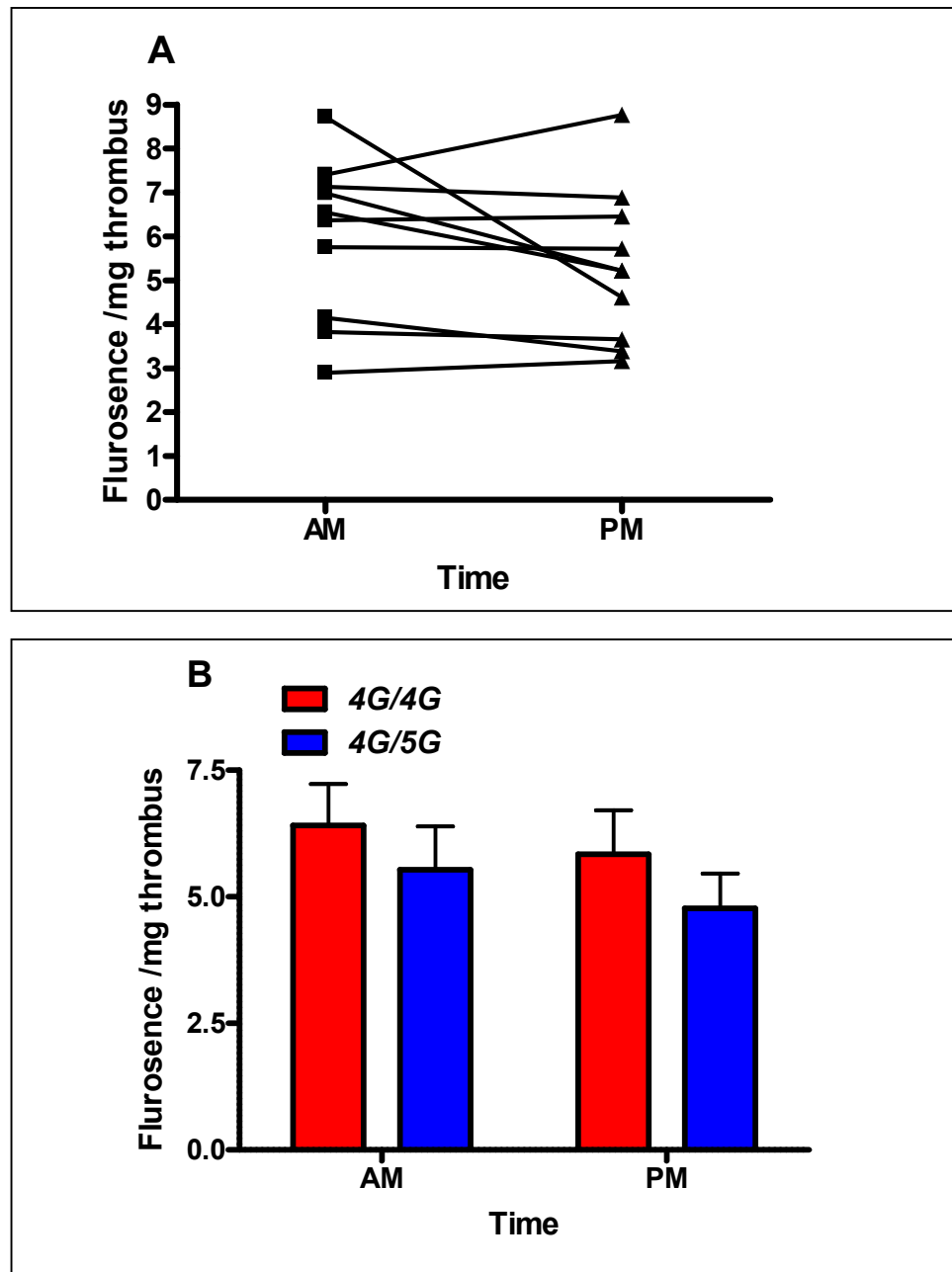


Figure 6.2 –Variation of thrombolysis as detected with Chandler loop (n=10; 5 of each genotype).

Thrombi were produced from whole blood of 10 volunteers in the Chandler loop at 9 am (AM) and 4 pm (PM). The rate of fibrinolysis was determined by the amount of FITC-fibrinogen released over 24 h. (A) Variation in thrombolysis between morning and evening time points are shown for each subject. (B) Time and genotype did not have a significant effect on thrombolysis. All values expressed as mean \pm SEM. Two-way ANOVA was used for statistical analysis.

However, T x G ($F_{(1,8)} = 7.12$, $P = 0.0283$) interaction did appear to have a significant effect on the weight of the thrombus, with larger clots produced from heterozygous donors than of *4G* homozygous subjects in both time points. These results indicate the type of genotype and time points interact in their effect on thrombus weight.

Thrombi were incubated in Hanks buffer for 24 h at 37°C, and the release of FITC-fibrinogen/fibrin was measured. Thrombolysis was expressed as the amount of FITC released from the thrombi relative to the weight of the thrombus at the start of the incubation. Time, Genotype or their interaction had a insignificant effect on clot lysis (Fig. 6.2, genotype $F_{(1,8)} = 0.88$, *ns*; for time $F_{(1,8)} = 1.81$, *ns*; for T x G interactions $F_{(1,8)} = 0.04$, *ns*).

6.2.2 – Thromboelastometry

Thrombi produced from Chandler loop mimic those formed under arterial flow conditions (Robbie *et al.* 1993; Stringer *et al.* 1994). To investigate the possible effect of circadian variation of PAI-1 levels on thrombi formed under venous flow conditions, thromboelastometry was employed. Blood was taken from the same 10 volunteers (five of each genotype *4G/4G* and *4G/5G*), at two time points; 9 am and 4 pm. Coagulation of 300 µl whole blood was initiated with 25µl 0.25M CaCl₂ and fibrinolysis enhanced with 5µl 0.175µg tPA.

TEM yields information relating to several components of clot formation; clotting time (CT, time from start of measurement until initiation of clotting), clot formation time (CFT, time from start of measurement until initiation of clotting), clot formation time (CFT, time from initiation of clotting until a clot firmness of 20 mm is detected) and

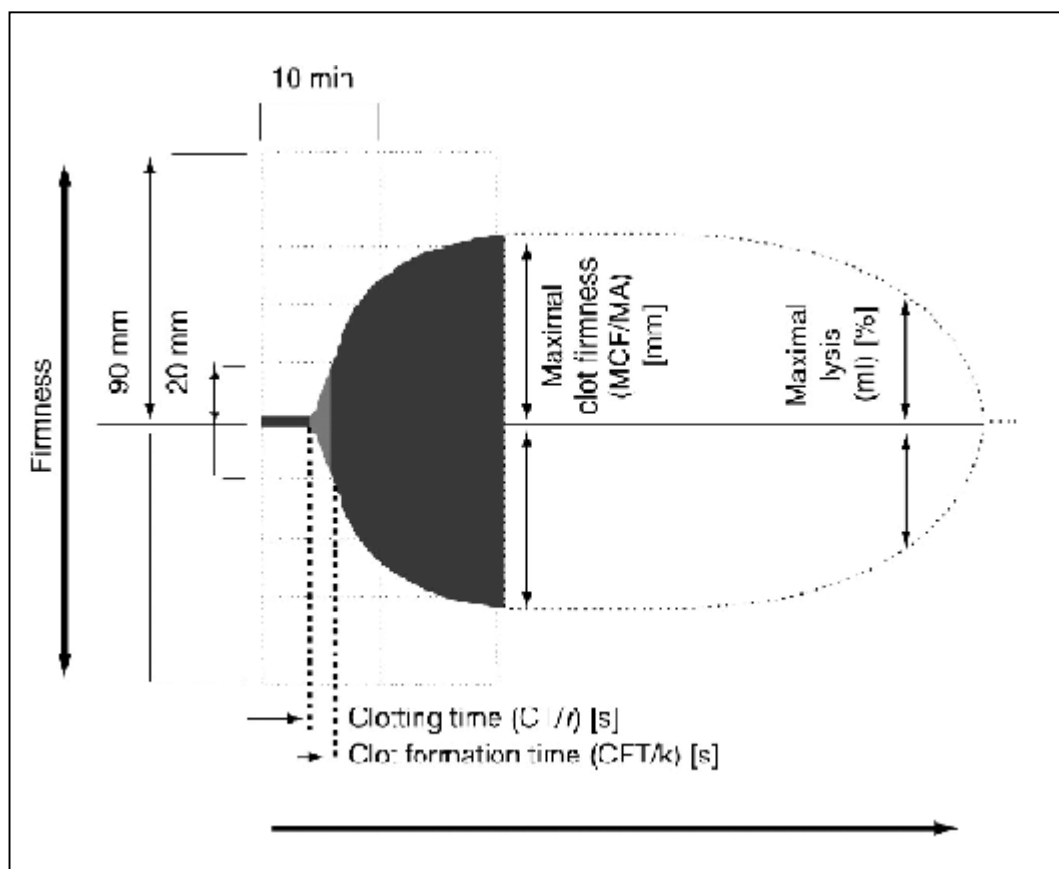


Figure 6.3 – Coagulation and fibrinolysis parameters shown in a typical thromboelastogram (adapted from Haizinger *et al.* 2006).

The following coagulation and fibrinolysis variables was measured in TEG at 37°C: clotting time (CT, initial light grey area), clot formation time (CFT, grey area) and maximal clot firmness (MCF, dark grey area).

maximal clot firmness (MCF, firmness of the clot) as seen in figure 6.3. Large variation in clot formation in terms of the clotting time, clot formation time, and maximal clot firmness (MCF) was observed between subjects (Fig. 6.4).

There was no significant genotype effect on CT (Fig. 6.5, $F_{(1,8)} = 2.91$, *ns*) and MCF ($F_{(1,8)} = 0.40$, *ns*). However, genotype was significant CFT ($F_{(1,8)} = 7.15$, $P = 0.0282$), with blood from 4G/5G heterozygotes forming clots more rapidly than that of 4G homozygotes. In addition, Bonferroni tests show significant differences in clot formation between genotype in the morning in CT and CFT ($P < 0.05$). T x G interaction were insignificant all clot formation parameters measured.

Fibrinolysis as assessed in TEG is described as the size of thrombi 60 minutes from after the MCF was recorded (Fig. 6.4). Increased fibrinolysis would therefore result in a decreased thrombus measurement. However, no significant effects were observed (Time, $F_{(1,8)} = 1.61$, Genotype, $F_{(1,8)} = 0.73$, T x G, $F_{(1,8)} = 0.08$).

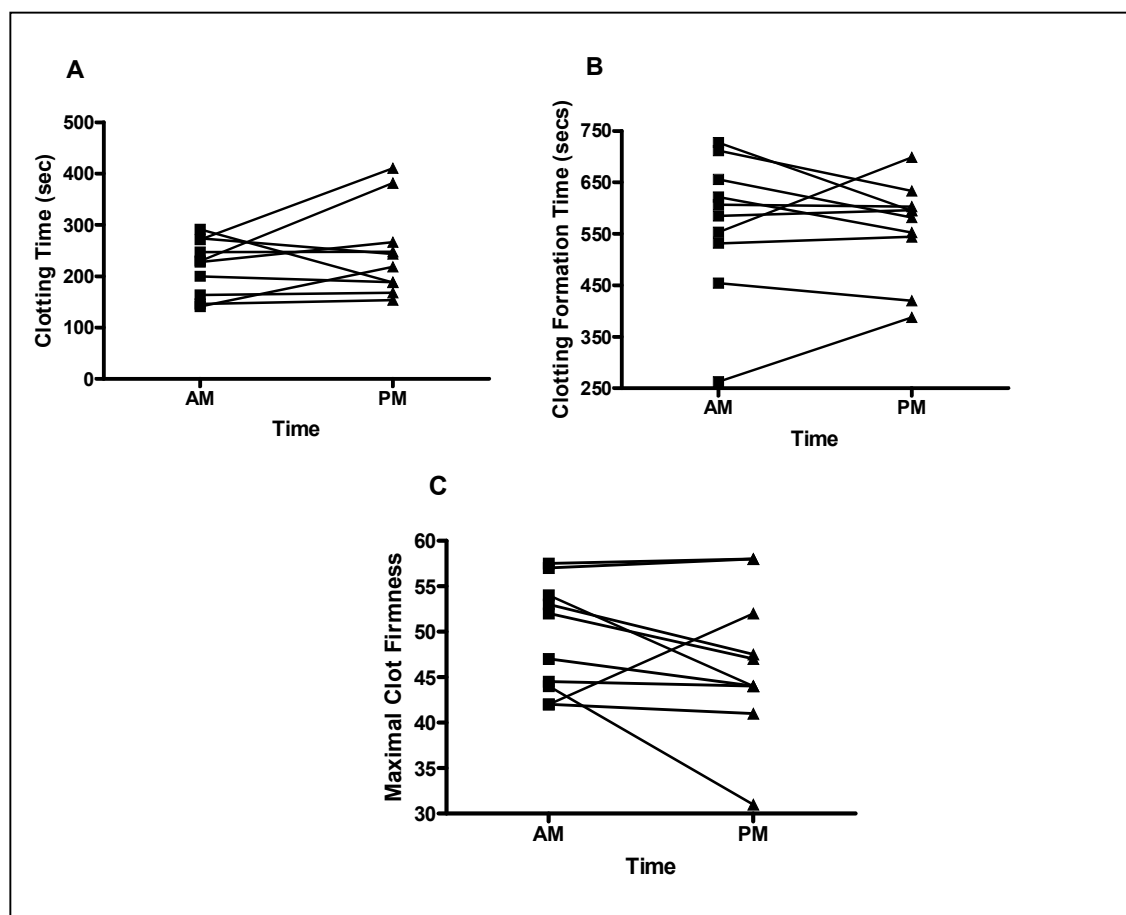


Figure 6.4 – Variation of clot formation as detected with TEM (n=10, 5 of each genotype).

Whole blood was taken from 10 volunteers at 9 am (AM) and 4 pm (PM) and various coagulation parameters were detected with TEM. Variation in CT (A), CFT (B) and MCF (C) between morning and evening time points are shown for each subject.

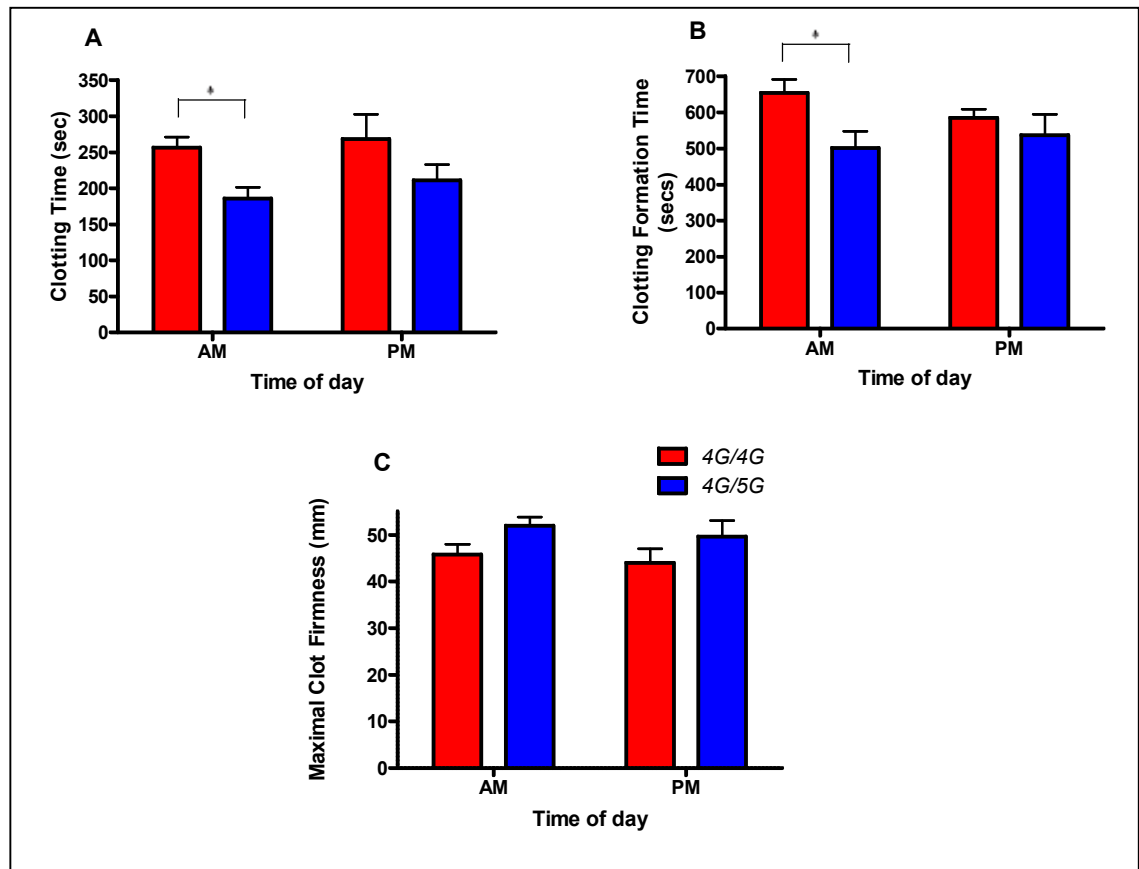


Figure 6.5 – Influence of the genotype with the diurnal variation of coagulation as detected with TEM (n=10, 5 of each genotype).

Whole blood was taken from 10 volunteers at 9 am (AM) and 4 pm (PM) and coagulation parameters was analysed for the potential influence with the 4G/5G polymorphism. Genotype had a significant effect on CT (A) but not CFT (B) or MCF (C). Time had an insignificant effect on all the coagulation parameters measured. $P < 0.05$ between 4G homozygous and heterozygous during AM time point. All values expressed as mean \pm SEM. Two-way ANOVA was used for statistical analysis.

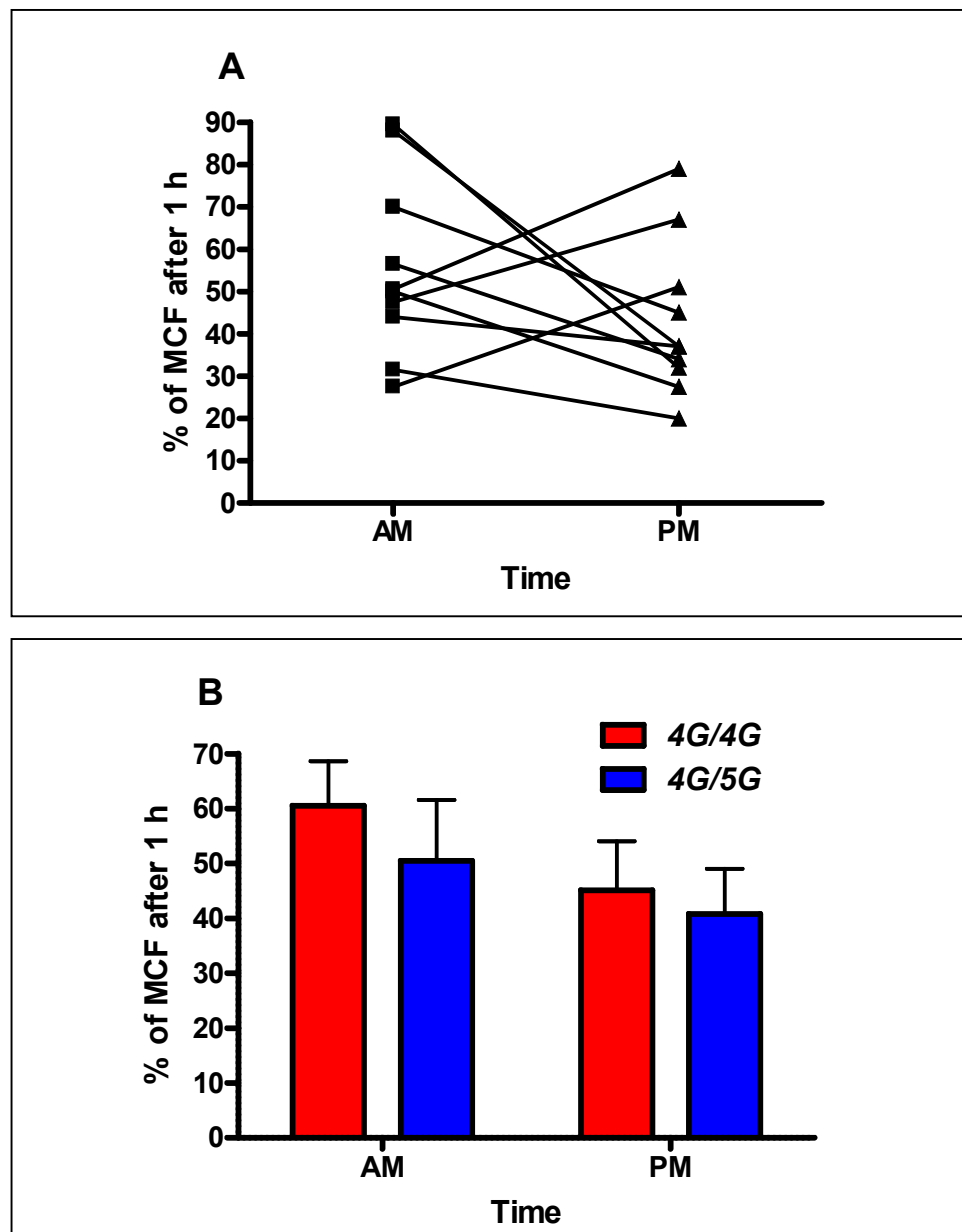


Figure 6.6 – Variation of thrombolysis as detected with TEM (n=10; 5 of each genotype).

Thrombi were produced from whole blood of 10 volunteers by TEG at 9 am (AM) and 4 pm (PM). The rate of fibrinolysis was determined by the size of the thrombus 1 h after maximal clot firmness was reached. (A) Variation in thrombolysis between morning and evening time points are shown for each subject. (B) Time and genotype did not have a significant effect on thrombolysis. All values expressed as mean \pm SEM. Two-way ANOVA was used for statistical analysis.

6.3 – Discussion

A number of haemostatic parameters has been shown to display circadian oscillations including; platelet aggregation (Haus *et al.* 1990; Jovicić and Mandić 1991), protein C (Undar *et al.* 1999), antithrombin (AT, Undar *et al.* 1999), factor VII (Kapiotis *et al.* 1997), protein S (Undar *et al.* 1999), tissue factor pathway inhibitor (TFPI, Pinotti *et al.* 1997), and fibrinogen (Pasqualetti *et al.* 1997; Kanabrocki *et al.* 1999). These observations together with data in chapter 4, showing platelets from 4G homozygous genotype contains and release significantly higher levels of PAI-1 than their heterozygous counter-parts, led to the hypothesis that lysis of clots produced from the same subjects would vary in a circadian manner, which would be also associated with the 4G/5G polymorphism.

In this study, diurnal variation and possible effect of the 4G/5G polymorphism was explored using 2 models to measure global fibrinolytic activity; Chandler loop (arterial model) and TEM (venous model). Thrombolysis as detected by the two methods differs between subjects, but however, failed to display diurnal variation or an effect of genotype. Previous reports have documented circadian rhythms in fibrinolytic activity. Rosing *et al.* (1970) reported a circadian rhythm in global fibrinolytic activity assessed by the fibrin plate assay applied to blood samples obtained from clinically healthy, non-fasting subjects. Recently, Westgate *et al.* (2008) reported key components of the molecular clock regulate the response to a thrombogenic stimulus using a mouse model. With the use of photochemical injury model in the mouse femoral artery, a diurnal variation in thrombotic vascular occlusion (TVO) was observed in WT mice. However,

mutation of core clock component (CLOCK^{mut}) resulted in loss of the temporal pattern in the susceptibility to TVO. The small number of volunteers in the current study might account for the discrepancies, along with the heterogeneity of the donors in terms of age, gender, BMI and ethnicity. The age range of subjects in the current study was 24-55 years, whereas Rosing *et al.* (1970) studied 19 to 30 years of age donors, suggesting that age may have an effect on the circadian variation of PAI-1. Previous studies have demonstrated gender differences in circulating PAI-1 levels, including the Framingham Offspring Study that showed lower levels of PAI-1 in females than males (Tofler *et al.* 1997; Asselbergs *et al.* 2006). A recent study by Naran and colleague (2008) demonstrated that PAI-1 concentrations was significantly higher in Indian and White than African subjects, which was also observed in an earlier study (Festa *et al.* 2003), reporting lower plasma PAI-1 concentrations in Afro-American than White subjects. Obesity, especially central fat, is associated with increased PAI-1 levels (Sartori *et al.* 2001), and furthermore, two groups have reported the effectiveness of weight reduction in lowering PAI-1 concentrations (Kockx *et al.* 1999; Mavri *et al.* 1999).

The examination of clot formation showed an unexpected association of the 4G homozygous genotype with longer clotting times and clot formation times. Thrombosis is a balance between coagulation and fibrinolysis, so this finding was surprising given that PAI-1 levels were higher in 4G homozygous subjects, which would favour a pro-thrombotic/antifibrinolytic environment, predicting that clotting time would be shorter in the 4G homozygous. This unexpected result could be an artefact, due to the small number studied, and while it is of interest, no hypothesis can be made until other coagulation parameter such as thrombin generation is studied.

6.4 – Conclusion

In conclusion, it is unclear from the present study whether thrombolysis either in terms of arterial or venous clot lysis is regulated by the circadian clock or the *4G/5G* polymorphism. This may be due to the small number of subjects or the parameters which affect circulating PAI-1 levels. Interestingly, it was found that the *4G/5G* polymorphism may play a role in thrombosis only during the morning time point. These results, together with the observations by Westgate and colleagues (2008) that diurnal variation in thrombogenesis was attenuated with the disruption in core clock components, suggest that the molecular clock may influence thrombosis through the regulation of PAI-1. However, given the high level of variation between subjects and the effects of components such as age and gender, these studies would need to be carried out in large cohorts of more defined phenotype.

Chapter 7

General Discussion

7.1 – Major findings in the thesis

Cardiovascular events resulting from diseases of the coronary arteries are the major cause of death and disability in the western world (reviewed in Petersen *et al.* 2004). The fibrinolytic system, which is composed of plasmin, the plasminogen activators, and their inhibitors, represent an important endogenous defence mechanism for the prevention of intravascular thrombosis. Decreased fibrinolytic activity as a result of increased PAI-1 concentration may play a role in pathogenesis of cardiovascular events, and indeed several studies have demonstrated that elevated levels of plasma PAI-1 was associated with increased risk of MI (Hamsten *et al.* 1985; 1987). The systemic plasma concentration of PAI-1 appears to be of limited importance for the regulation of the local availability of active tPA (Hrafnkelsdottir *et al.* 2004). This is particularly so under stimulated conditions when the secretion rate of total tPA by far out weighs the level of circulating PAI-1 (Hrafnkelsdottir *et al.* 2004). Therefore, measurement of the plasma levels of tPA and PAI-1 may not necessarily reflect fibrinolytic activity at the local level. This thesis investigated the regulation of PAI-1 levels produced by cells of the circulatory system, specifically monocytes and platelets, and how this may be influenced by circadian factors and/or 4G/5G polymorphism, and to assess the possible effect of monocytes and platelet PAI-1 expression on clot lysis in 2 global measures of haemostasis. The major findings of the present study are: a) the production of *Pai-1* mRNA by monocytes is independent of both circadian gene expression and the 4G/5G polymorphism; b) platelets induce significant amounts of PAI-1 in monocytes to a much higher level than a inflammatory stimulus such as LPS, and that platelet stimulation of monocytes is principally through secreted factors, predominantly TGF- β 1 (both

exogenous and endogenous); c) platelets contain PAI-1 protein that reflect levels in the plasma with respect to *4G/5G* polymorphism; d) platelets contain *Pai-1* mRNA, but it is unclear whether platelets can synthesise PAI-1 protein. The present findings indicate PAI-1 in circulatory cells is regulated by circadian factors under resting conditions, but circadian influences may be masked following stimulation, whether inflammatory (LPS) or thrombotic. The *4G/5G* polymorphism appears to regulate the amount of PAI-1 released by stimulated whole blood, with platelets as possibly the main source.

7.2 – Limitations of the study

The present study has some limitations. First, the findings in the thesis excluded the endothelial response. The interaction between endothelial cells, platelets and monocytes lead to complex changes that results in atherosclerosis, intimal hyperplasia and acute thrombosis. Human endothelial cells are the major site of synthesis of PAI-1, and are one of the potential sources of plasma PAI-1. Furthermore, the *4G/5G* polymorphism appears to play a role in the regulation of PAI-1 in stimulated endothelial cells. This was evident in previous studies demonstrating that the polymorphism mediated PAI-1 induction by TNF α (Swiatkowska *et al.* 2005), IL-1 (Ronai *et al.* 2006) and angiotensin II (Ronai *et al.* 2004). Therefore to get the overall picture of the influence of the *4G/5G* polymorphism and circadian clock on thrombolysis, the contribution of endothelial cells cannot be ignored. Second, the cohort size in the present study (10 donors) was too low to conclude the effect of the *4G/5G* polymorphism on PAI-1 regulation with certainty. To fully justify the findings in the present study, a larger sample size is essential. Additionally, a bigger cohort will allow for examination of other factors that may affect

PAI-1 levels, such as sex, BMI and ethnicity. Furthermore, this study was carried out on the assumption that the regulation of PAI-1 in heterozygous genotype is similar to that of 5G homozygous genotype. Further investigations needs to be carried out on 5G homozygous subjects to investigate if this assumption is valid. Third, the experiments carried out to investigate the circadian regulation of PAI-1 were limited by the number of time points used in this study. To further examine the influence of the circadian clock on the regulation of PAI-1, increasing the number of time points in terms sample collection and stimulation experiments is essential. Nevertheless, the main finding of the present study was the monocyte response to platelets following activation.

7.3 – Regulation of monocytes PAI-1 by activated platelets

Monocytes contribute to inflammation by supplying tissues with macrophages and dendritic cell precursors (reviewed in Serbina *et al.* 2008) and play a role in thrombosis through their interaction with platelets. But which stimuli might elicit *Pai-1* gene expression in monocytes? Resting monocytes express little PAI-1, however, the present study found that following activation by CRP-XL, platelets potently stimulate *Pai-1* gene expression in human monocytes. Furthermore, monocytes did not express significant levels of *Pai-1* mRNA in response to bacterial endotoxin (LPS). The increased responsiveness of monocyte PAI-1 expression to a thrombotic stimulus highlights the importance of PAI-1 produced by monocytes in creating a pro-thrombotic environment.

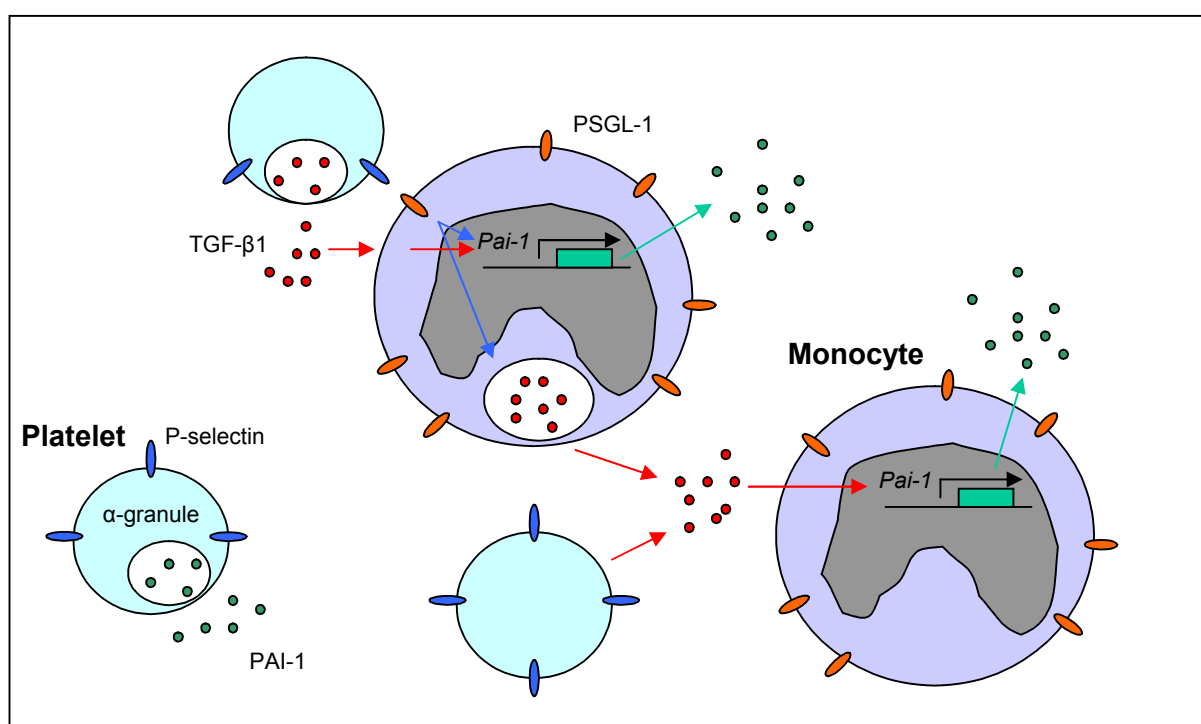


Figure 7.1 – Proposed role of TGF-β1 in the regulation of monocyte PAI-1

Platelet activation results in the acute release of PAI-1 from their α-granules. In addition, TGF-β1 is also released and subsequently induces monocyte PAI-1 expression in a paracrine mechanism. Stimulated monocytes can also release TGF-β1 increasing the local level, and inducing the expression of monocyte PAI-1 in an autocrine manner.

Studies carried out in the current investigation have identified two potential mechanisms responsible for the induction of monocyte PAI-1 by activated platelets (Fig. 7.1). A positive, paracrine regulation of PAI-1 was described, which appeared to be primarily mediated through TGF- β 1. Another putative signalling route is a cell surface-based signalling system; P-selectin binding to its receptor PSGL-1 on the leukocyte, that can induce *Pai-1* expression, mediated again via TGF- β 1, but in an autocrine manner. Because the interaction between platelets and monocytes is essential for thrombosis, these novel pathways may contribute to monocyte PAI-1 expression in the human thrombus. The inhibition of PAI-1 maybe a potential therapeutic strategy in the prevention of thrombus formation, indeed inhibition of PAI-1 activity by monoclonal or polyclonal antibodies prevents thrombosis (Ahrahamsson *et al.* 1996; van Giezein *et al.* 1998). Several small molecule weight compounds have been reported, which inhibit either PAI-1 production or activity such as compound ZK4044, acting by directly binding to PAI-1 thus preventing PAI-1 binding to tPA (Liang *et al.* 2005). However, the concern of suppressing the action of total PAI-1 is the danger of excessive bleeding following injury. Data from the present study suggest that it may be possible or even advantageous to exploit TGF- β 1 as a means of lowering PAI-1 levels. Targeting TGF- β 1 would not inhibit the acute release of PAI-1 by activated platelets, but would lower the amount of PAI-1 produced by monocytes resulting in a net reduction in total level of PAI-1 within a thrombus. This may present a better alternative in therapeutic design than directly targeting PAI-1, and may lead to a useful treatment for cardiovascular events.

Several large and small molecule drugs inhibiting TGF- β are being examined which include mAbs (Lim and Zhu 2006; Liu *et al.* 2006; reviewed in Biswas *et al.* 2007), soluble TGF- β receptors (Breitkopf *et al.* 2005; Lim and Zhu 2006; reviewed in Liu *et al.* 2006), antisense oligonucleotides (ODNs; reviewed in Liu *et al.* 2006; Wick *et al.* 2006; Lim and Zhu 2006) and inhibitors of TGF- β RI (de Gouville and Huet 2006; reviewed in Tsuchida *et al.* 2006; Liu *et al.* 2006; Wick *et al.* 2006). These TGF- β suppressing drugs are currently being produced primarily to treat either fibrotic disease or cancer. In terms of cardiovascular disease, antihypertensive drugs that block the RAS system such as ACE inhibitors, and antagonists of the angiotensinII type 1 receptor (AT1) can inhibit TGF- β production (Wolf 2006; Bujak and Frangogiannis 2007). This may be due to the fact that angiotensinII (AngII) stimulates TGF- β production (Wolf 2006; Bujak and Frangogiannis 2007), and also enhances the expression of the TGF- β receptors through the MAPK pathway (Mehta and Attramadal 2007). Previous studies also found that AngII up-regulates TSP-1 production and TSP-1 dependent TGF- β activation in rat cardiac fibroblasts (Zhou *et al.* 2006). TSP-1, as mentioned in section 3, activates latent TGF- β . The group also demonstrated AngII dependent effects were blocked by Losartan, an AngII receptor blocker. Another important inhibitor of TGF- β in relation to cardiovascular system is the anti-inflammatory drug tranilast. Developed by Kissei Pharma in Japan (Konneh 1998), this has been used clinically in Japan and South Korea for the treatment of allergic disorders (Okuda *et al.* 1984; Konneh 1998) and has potent antifibrotic effects (Konneh 1998; Suzawa *et al.* 1992), which may stem from its ability to inhibit both TGF- β secretion and action (Martin *et al.* 2005; Suzawa *et al.* 1992). Additionally, tranilast have been shown to inhibit both TGF- β secretion and TGF- β receptor expression in cell culture assays (Munger *et al.*

1999; Ward *et al.* 1998). These less specific drugs might be more advantageous than using more specific ones, because they target other aspects of the pathological process and are generally less toxic.

7.4 – Regulation of monocyte PAI-1 is independent of peripheral circadian clock

Circulating plasma PAI-1 and its parallel activity are characterised by highly reproducible high-amplitude circadian rhythmicity (Andreotti and Kluft 1991). This circadian rhythm overrides the circadian variation in circulating tPA activity and determines the 24-hour variation in overall fibrinolytic activity (Andreotti and Kluft 1991; Angleton *et al.* 1989; Andreotti *et al.* 1988). It has been suggested that the source of plasma PAI-1 originate from various cell types including hepatocyte, adipocyte and endothelial cells. PAI-1 production in vascular endothelial cells in vitro (Schoenhard *et al.* 2003; Maemura *et al.* 2003; Schoenhard *et al.* 2002), and in vivo (Mohri *et al.* 2003) is driven by fundamental circadian clock components. In transfected endothelial cells, both CLOCK:BMAL1 and CLOCK:BMAL2 heterodimers activate PAI-1 gene transcription (Chong *et al.* 2006; Schoenhard *et al.* 2003). But is PAI-1 expression in monocytes regulated by a peripheral circadian clock? *Pai-1* expression appears to be regulated by circadian clock in monocytes under basal conditions. However, the regulation of PAI-1 in monocytes differs substantially in stimulated conditions. The expressions of most clock genes investigated were either down-regulated or unaffected in monocytes in response to either an inflammatory or thrombotic stimuli. Hence, the acute induction of monocyte PAI-1 appears to be independent of the peripheral circadian clock. To examine this hypothesis further, and to demonstrate whether the

circadian clock component directly regulate PAI-1 expression in monocytes, two methods could be utilised, EMSA or chromatin immunoprecipitation assay (ChIP).

EMSA involves extracting proteins from lysed monocytes and test whether the proteins binds to a labelled oligonucleotide probe contain the E-boxes from the PAI-1 promoter (as described in the introduction section). If binding between monocyte proteins and the probe occurs, the resulting complex would be less mobile than the unbound probe, thus shifted up on the agarose or polyacrylamide gel. To examine if the clock components are bound to the complex, an antibody that recognises BMAL1/2 or CLOCK could be added to the mixture to produce a larger complex resulting in a greater shift, this method is known as supershift assay. The disadvantage of EMSA is that one can only study *in vitro* protein-DNA interactions. However, ChIP assay could be used to study the *in vivo* interaction between the clock components and the E-boxes in the PAI-1 promoter. The principle underpinning this assay is that DNA-bound protein (CLOCK/BMAL1/2-*pai-1*) can be cross-linked to the chromatin. This is usually accomplished by gentle formaldehyde fixation. Following fixation, monocytes are lysed and the DNA is broken into pieces 0.2-1 kb in length by sonication. Once the proteins are immobilized on the chromatin and the chromatin is fragmented, CLOCK/BMAL1-*pai-1* complexes can be immunoprecipitated using a specific antibody. The DNA from the isolated protein/DNA fraction can then be purified. The identity of *pai-1* fragments isolated in complex with the CLOCK/BMAL1 can then be determined by PCR using primers specific for the *pai-1* gene. Furthermore by using specific primers for E-box one or two, enrichment of CLOCK/BMAL1 onto these two E-boxes could be investigated. The interaction between Smads proteins with the clock heterodimers might

occur to regulate stimulated monocyte PAI-1 expression, as the same E-box was demonstrated to be essential for the activation of *Pai-1* promoter (discussed in the introduction section; Chong *et al.* 2006; Hua *et al.* 1999). To test the potential circadian-dependent PAI-1 response to TGF- β 1, protein/DNA complex could be isolated from monocytes at different times of the day, and the enrichment of SMAD proteins to the *Pai-1* promoter E-boxes determined by using antibodies specific for these proteins.

7.5 – Monocyte peripheral circadian clock

Previous reports have shown the circadian expression of clock genes in both whole blood and lymphocyte preparations (Boivin *et al.* 2003; Teboul, *et al.* 2005; Fukuya *et al.* 2007). Consistent with these reports, the present study demonstrated expression of clock genes in monocytes, suggesting that a peripheral circadian clock does exist in this cell type. However, the observation of attenuated or unaffected clock gene expression in stimulated monocytes leads to the question why monocytes contain a circadian oscillator? A clue to answering this question comes from the data showing monocyte *mPer2* expression being up-regulated by CRP-XL stimulated platelets. The induction of *mPer2* expression has been reported to be involved in the synchronisation of the circadian clock (Albrecht *et al.* 2001; Shearman *et al.* 1997, Shigeyoshi *et al.* 1997). This raises the possibility that the circadian clock may take part in the regulation of processes involved during monocyte stimulation in a thrombotic environment. One method of studying which genes or pathway may be regulated by the circadian clock in monocytes is use a combination of ChIP and whole-genome DNA microarrays (ChIP on chip), which yields high-resolution, genome-wide maps of the *in vivo* interactions

between proteins and DNA. The procedure for ChIP is as described in the last section (6.3), except following the reverse cross linking and purification, the isolated DNA is hybridised to a DNA microarray containing elements that represent the entire genome. The results of the hybridisation allow one to identify which segments of the genome were enriched with the CLOCK/BMAL1/2 proteins. Since the precise location of each arrayed element is known, construction of a genome-wide map of *in vivo* CLOCK/BMAL1/2-DNA interactions in monocyte is possible, and thus mapping out the regulatory pathways regulated by the circadian clock in monocytes.

In summary, monocytes may play a role in thrombosis through the expression of PAI-1. The data produced in this study emphasize the importance of platelets in provoking this response. In addition, platelets can also contribute in elevating PAI-1 levels by releasing and synthesising new PAI-1 protein. Furthermore, circadian factors appear to regulate PAI-1 expression of cells under basal conditions, but independent during stimulated environment. The 4G/5G polymorphism appears to regulate the amount of PAI-1 released by stimulated whole blood. However, whether circadian factors and/or the 4G/5G polymorphism influence the formation or resolution of a thrombus remains to be elucidated.

Chapter 8

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8.0 – References

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Chapter 9

Appendix

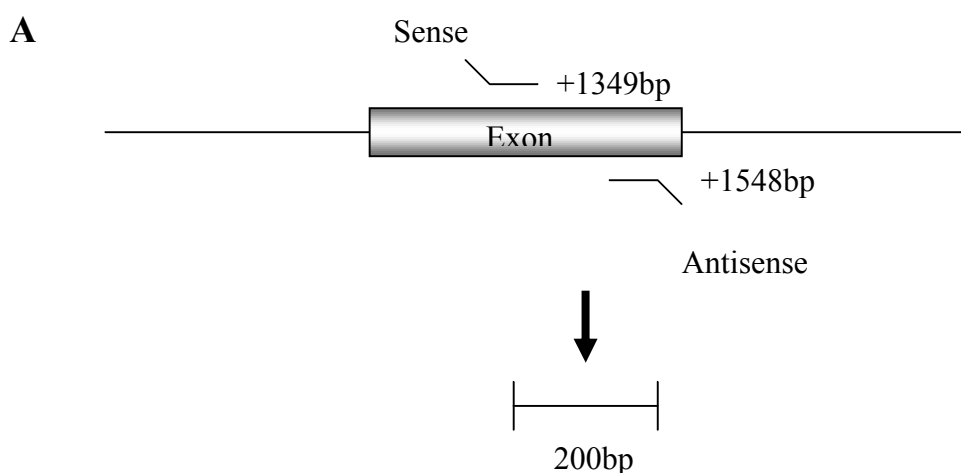
9.1 – Optimisation of semi-quantitative PCR

To study the expression of *Pai-1* and the core clock genes, various parameters were examined to produce the optimal PCR condition, including cycle number, primer design and template concentration. The cDNA used for the optimisation process was prepared from monocytes that had been stimulated with LPS for 4 h.

9.1.1 – Optimising cycle number for *18s* and *Pai-1*

The number of amplification cycles necessary to produce a band visible on a gel depends largely on the starting concentration of the target DNA and the gene under investigation. Additionally, the number of cycles for amplification must not be too high as to allow the PCR products to become saturated. The opposite is also true, where the cycle number must not be too low to produce the minimal amount of PCR product that can only just be seen on a gel.

The intensity of the *18s* amplicons could be seen to gradually increase from 24 to 26 cycles (Fig. 9.1). The PCR amplification then increased linearly from 27 to 32 cycles. For *18s*, the most appropriate number of amplification to use was 30 cycles, which fell in the mid-point of the curve (Fig. 9.1). Choosing the most suitable cycle number for *Pai-1* was more difficult, as the exponential phase occurred over a smaller range (Fig. 9.2). However, 34 cycles was chosen in preference to 35 cycles, because reducing the cycle number increased the specificity of the PCR reaction. The same procedure was also performed for *Bmal1*, *Bmal2*, *Dec1*, *mPer2* and *Clock* and the optimal cycle number for each gene is shown in table 3.1.



Primer	No. Of Base Pairs	GC Content (%)	Melting Tempertaure (°C)	Sequence (5' to 3')
Sense	20	50	55.5	GTGGAGCCATTGTGTTGGTT
Antisense	20	60	57.5	CGCTGAGCCAGTCAGTGTAG

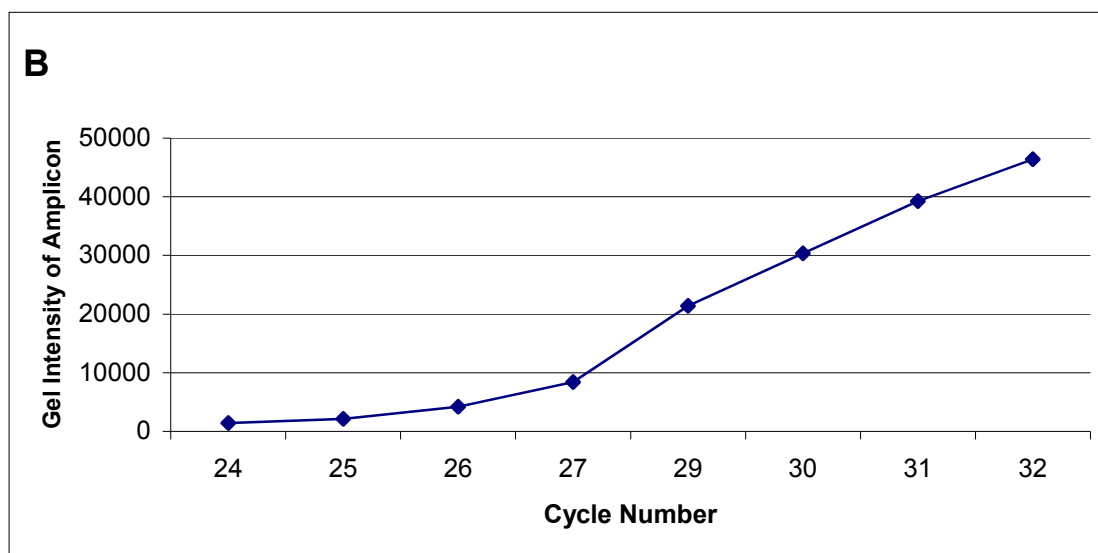
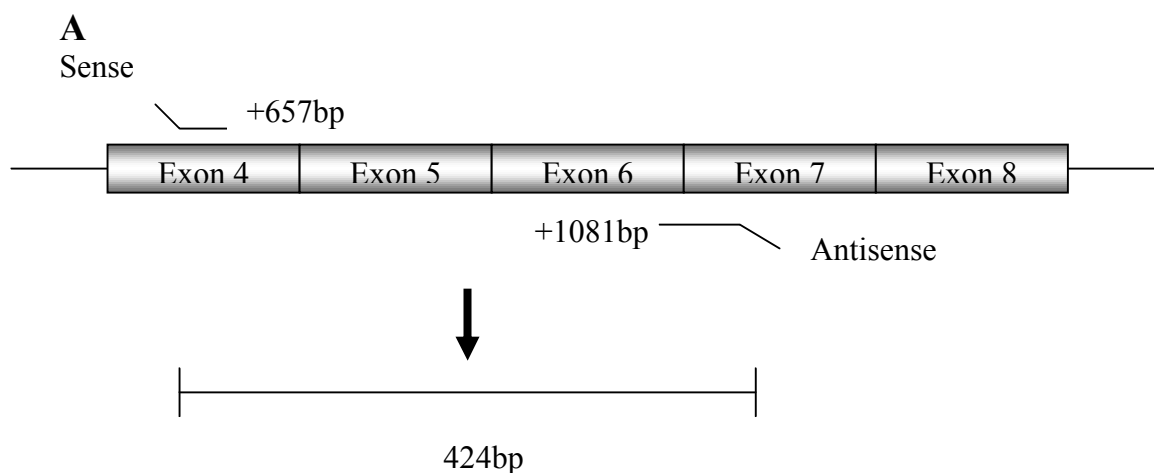


Figure 9.1 – Optimisation of 18s for semi-quantitative PCR.

A. Schematic diagram of primers binding on 18s. The PCR amplifies the region +1349bp to +1548bp from the transcriptional start site, resulting in a 200bp product. B. Characteristic of primers, C. Gel intensity of 18s amplicons in relation to cycle number.



Primer	Length	GC Content (%)	Melting Temperature (°C)	Sequence (5' to 3')
Sense	20	60	58.3	CAACGGCCAGTGGAAGACTC
Antisense	23	47.8	55.4	CTTGGTCTGAAAGACTCGTGAAG

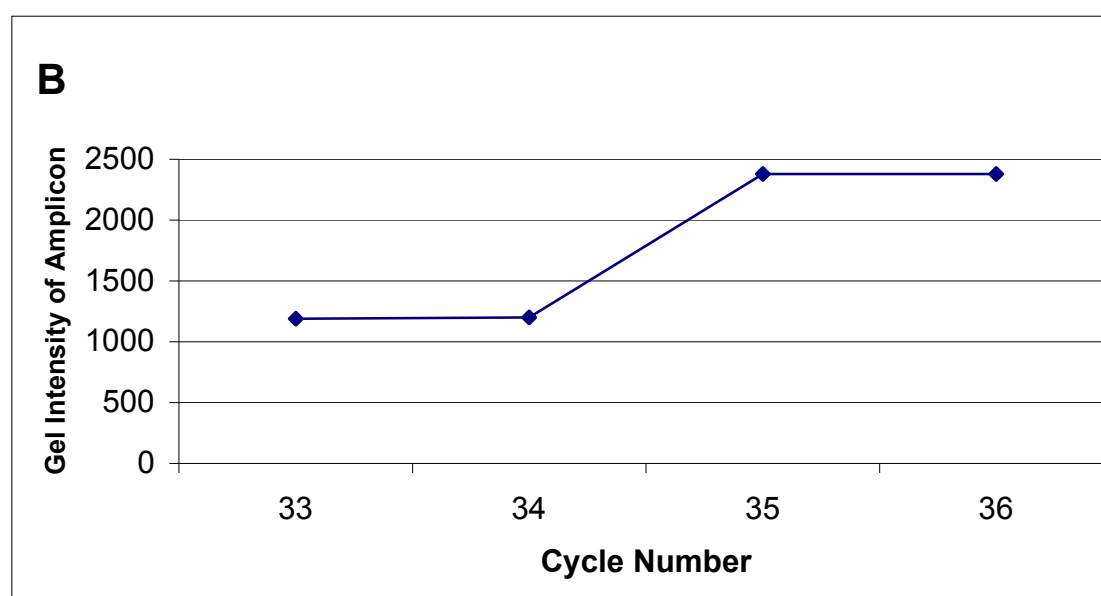


Figure 9.2 –Optimisation of *Pai-1* for semi-quantitative PCR.

A. Schematic diagram of Set 2 primers binding on *Pai-1* (A). The PCR amplifies the region +657bp to +1081bp from the transcriptional start site, resulting in a 424bp product. B. Characteristic of primers, C. Gel intensity of *Pai-1* amplicons in relation to cycle number.

9.1.2 – Optimising *Pai-1* primer pairs

One of the most critical parameters for successful PCR is the design of primers. A poorly designed primer can result in little or no product due to non-specific amplification and/or primer-dimer formation, which can become competitive enough to suppress product formation. It is well established that the 3' terminal position in PCR primers is essential for the control of mis-priming (Kwok et al., 1990). Most of the primers designed in this study were “stickier” on their 5' ends than on their 3' ends. A sticky end was indicated by a high G/C content.

Gene	Control	CRP-XL	LPS
<i>18s</i>	29	29	29
<i>Pai-1</i>	36	30	33
<i>Dec1</i>	27	24	24
<i>Bmal2</i>	31	31	31
<i>Bmal1</i>	29	29	29
<i>mPer2</i>	30	30	30
<i>Clock</i>	30	30	30

Table 9.1 – Optimal cycle numbers used to amplify the various genes.

The optimal number of amplification cycles for each gene was found to vary depending on the stimulus, where CRP-XL were monocytes stimulated with cross-linked collagen-related peptide, and LPS were monocytes incubated with lipopolysaccharides. Monocytes were stimulated by both agonists for 4 h at 37°C.

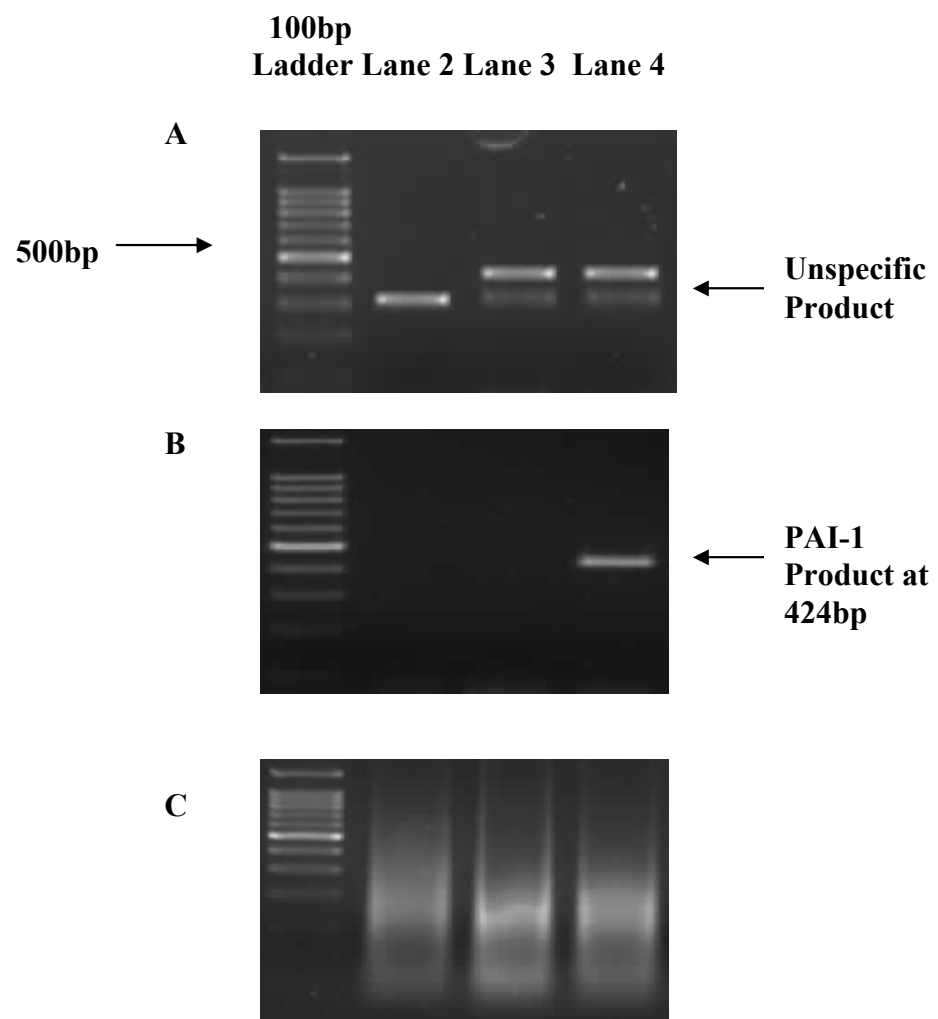


Figure 9.3 – *Pai-1* optimisation of *Pai-1* primers for semi-quantitative PCR.

A. *Pai-1* amplified with primer set 1 (10ng template). B. *Pai-1* amplified with primer set 2 (10ng template). C. *Pai-1* amplified with primer set 2 (20ng template). Lane 1 is 100bp ladder, lane 2 is the amplicon resulting from amplifying mRNA in monocytes that were extracted immediately, lane 3 is the amplicons yielding from mRNA in monocytes that were incubated at 37°C for 4 h without stimulation, lane 4 is the amplicon from mRNA in monocytes that were incubated at 37°C for 4 h with LPS.

The first set of primers designed for *Pai-1* had a “sticky” 3′ end and produced a single band of 300bp representing the *Pai-1* amplicon as can be seen in lane 2 (Fig. 9.3 – mRNA from monocytes that had not been stimulated). The same PCR product could be seen in mRNA amplified from monocytes that had been incubated at 37°C for 4hrs (lane 3), or for 4hrs with LPS (lane 4). However, an extra PCR product was also observed in these two lanes of approximately 350bp (Fig. 9.3 A). This was due to the primer annealing at multiple sites on the template DNA. The *Pai-1* primers were re-designed to have a “stickier” 5′ end, which resulted in a more specific PCR reaction that yielded only one band of 450bp (Fig. 9.3 B). When possible, a “G” or “C” was included at the 3′ end (termed GC clamp) to reduce spurious secondary bands (Sheffield et al., 1989).

9.1.3 – Optimising template DNA concentration

The concentration of template DNA can also affect the efficiency and specificity of a PCR reaction. Inadequate concentration of DNA would result in the PCR especially prone to PCR induced artefacts. In an effort to improve the efficiency of the PCR, the template concentration was increased from 10ng to 20ng (1μl to 2μl) in the PCR reactions and resulted in the smearing of bands when subjected to gel electrophoresis. This occurred in all three lanes shown in Fig. 9.3 C, where lane 2 is the amplicon resulting from amplifying mRNA in monocytes that were extracted immediately, lane 3 is the amplicons yielding from amplifying mRNA in monocytes that were incubated at 37°C for 4hrs, lane 4 is the amplicon produced amplifying mRNA from monocytes that were stimulated by LPS and incubated at 37°C for 4hrs. These results may be due to the primer/template ratio being too high, which results in a higher probability to generate

non-specific amplification products. Therefore the most suitable template concentration to use in a PCR reaction for *Pai-1* was 10ng.

9.2 – Optimisation of real-time PCR

The sensitivity of semi-quantitative PCR was limited, which could cause problems in quantification of *Pai-1* mRNA abundance. To increase the sensitivity of the PCR reaction, real-time PCR was used. For optimising real-time PCR conditions cDNA was prepared from monocytes that had been stimulated with LPS for 4 h.

9.2.1 – SYBR-Green method

A real-time PCR amplification plot is given as the number of PCR cycles (cycle threshold or Ct) necessary to achieve a given level of fluorescence. During the initial PCR cycles, the fluorescence signal emitted by SYBR-Green I bound to PCR product was usually too weak to register above background. A difference could not be seen until after about 25 PCR cycles for *18s*, and 35 cycles for *Pai-1*. After 35 cycles, the intensity of fluorescence signal for *18s* began to plateau, indicating that the PCR had reached saturation. This was not observed for *Pai-1*. For this study, the Ct was fixed in the exponential phase of the PCR where the fluorescence doubled at each cycle, at 0.2 (Fig. 9.4). This lies in the log-linear phase for both PCR reactions, which were 27.2 and 36.0 for *18s* and *Pai-1* respectively. This indicates that *18s* was relatively more abundant than *Pai-1* mRNA in stimulated monocytes. After the amplification process, dissociation curves were used to provide a graphical representation of the PCR product. A single peak was observed for *18s* amplification with a melting temperature of 86.5°C,

however *Pai-1* produced several peaks, one main peak at 87°C, and a few smaller peaks at 80.5°C, 77°C and 74°C (Fig. 9.4). The PCR products were further confirmed by gel electrophoresis. The *18s* PCR reaction produced one 200bp band, whereas *Pai-1* product gave variable appearances in the gel, sometimes resulting in a smear around 450bp (Fig. 9.4).

The optimisation of template concentration is essential, as explained in section 9.1.3. A range of concentrations of template DNA was used for *Pai-1* PCR (table 3.2, Fig. 9.5), after non-specific products were found in *Pai-1* PCR. High template concentration, resulted in less *Pai-1* PCR product formed, and more non-specific products produced. The lower template concentration resulted in primer-dimers, which was due to the low abundance of *Pai-1* transcripts.

Template Concentration (ng)	<i>Pai-1</i> Average Ct Value
2.5	37.6
5	36.0
10	34.5
20	29.7
30	29.3

Table 9.2 – Ct values for *Pai-1* PCR at different template concentration

The Ct values to reach exponential phase of *Pai-1* PCR with various template concentration.

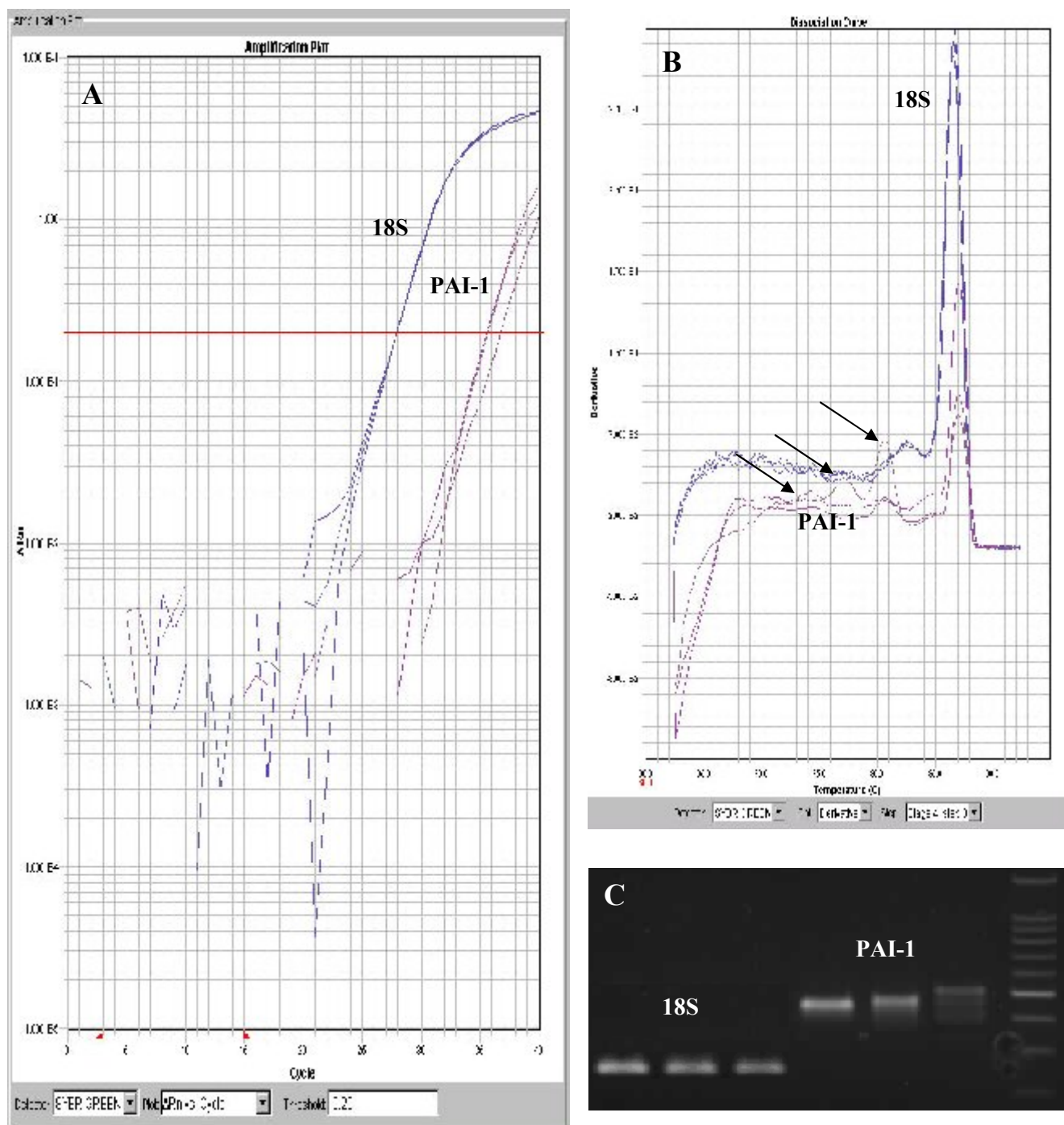


Figure 9.4 – Optimisation of real time PCR for *Pai-1* and *18s* using the SYBR-green method.

(A) Three real time amplification plots for *Pai-1* and *18s*. The Ct was set as 0.2 (horizontal red line) for both PCR reactions (vertical line). (B) Dissociation curves for *Pai-1* and *18s* PCR reactions. A single peak was seen for the *18s* PCR. The *Pai-1* PCR resulted in 3 extra peaks (as shown by the arrows). (C) Gel electrophoresis of the PCR products from the 3 reactions for *18s* and *Pai-1*.

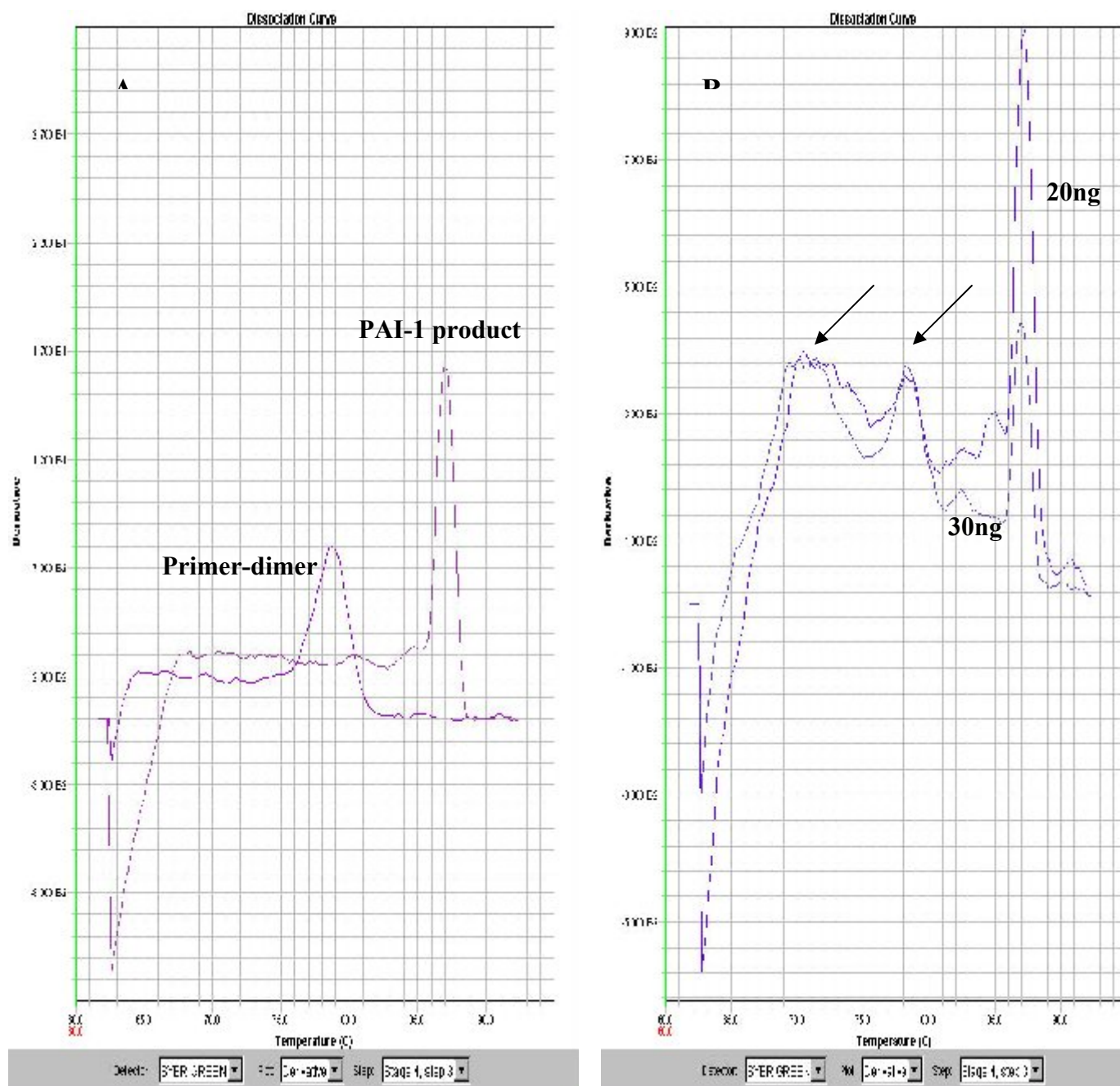


Figure 9.5 – Effect of template concentration on *Pai-1* dissociation curves using the SYBR-green method.

Dissociation curves showing the results of varying template concentrations. (A) Using 2.5 ng template resulted in primer-dimers. (B) Using 20 ng and 30 ng produced non-specific products (arrows).

9.2.2 – TaqMan method

Taking the results together so far, it appears that the SYBR-Green I method lacked sensitivity for *Pai-1* mRNA in stimulated monocytes; no plateau phase was reached in the *Pai-1* PCR reaction, and the *Pai-1* PCR sometimes produced non-specific products. To increase the level of sensitivity, the TaqMan method was employed. The 5' nuclease activity is based on the specific hybridisation of a dual labelled TaqMan. The detection method is the same as SYBR-Green I. The exponential phase of the PCR occurred at 22.7 and 37 cycles for *18s* and *Pai-1* respectively, suggesting again that *18s* expression is higher than *Pai-1*. The plateau phase was reached after 30 cycles in *18s* PCR, and 44 cycles for *Pai-1*. The Ct value of *Pai-1* were similar in both the TaqMan and SYBR-Green I methods indicate that the sensitivity of *Pai-1* PCR have not increased with the TaqMan approach. However, the additional level of specificity introduced by the hybridisation of the PCR product to a specific fluorescent TaqMan probe was evident. The PCR products were further confirmed by gel electrophoresis. The *18s* PCR reaction produced one 200bp band, and *Pai-1* yielded a single 100bp band (Fig. 9.6). No additional bands can be observed other than the product band, indicating no primer-dimer nor non-specific product. Due to this additional level of specificity no further optimisation was required.

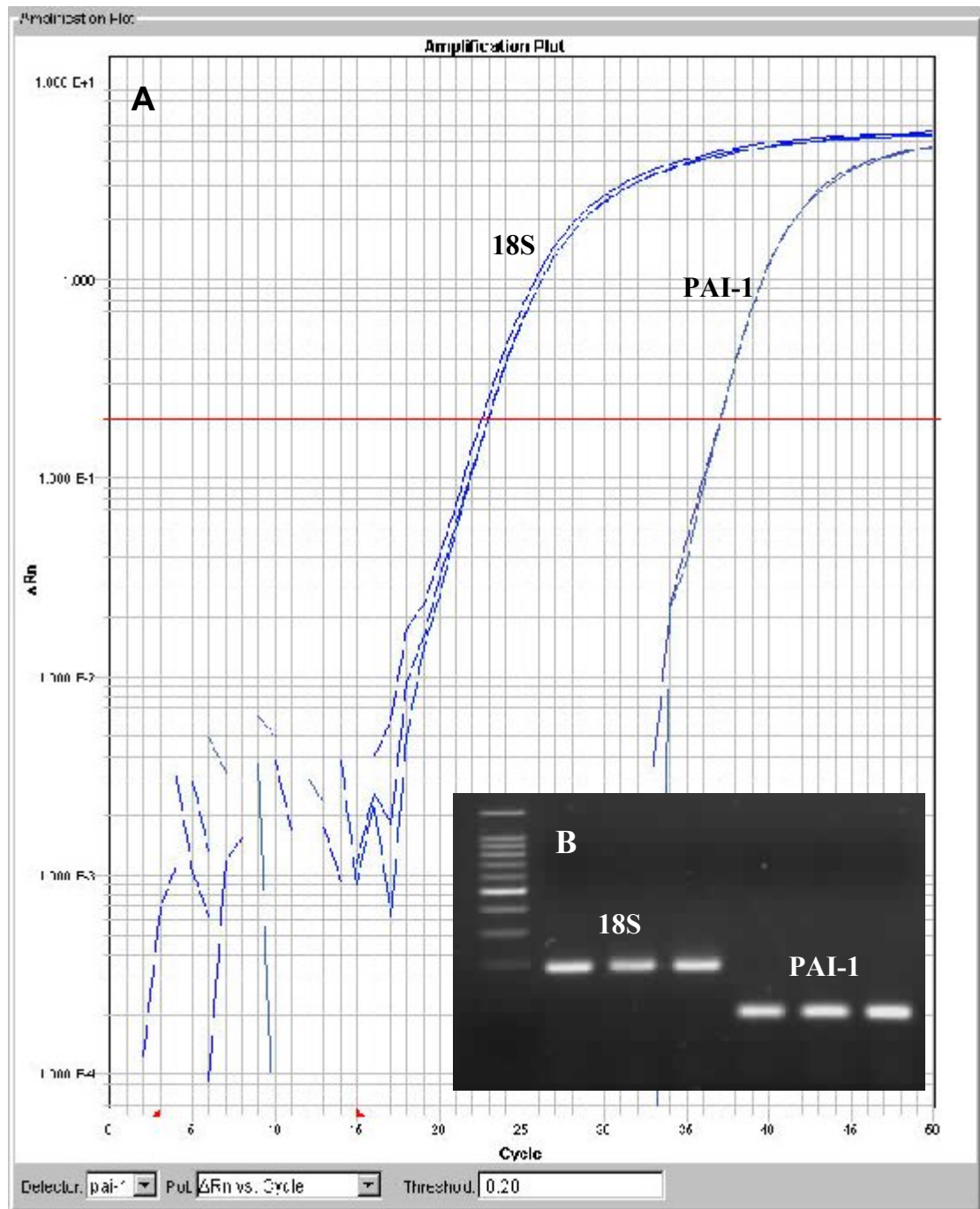


Figure 9.6 – Optimisation of real-time PCR for *18s* and *Pai-1* using Taqman method.

A. Three amplification plots for *Pai-1* and *18s*. DNA from monocytes stimulated with LPS for 4hrs was used for this experiment. The Ct was set as 0.2 (horizontal red line) for both PCR reactions (vertical line). B. Gel electrophoresis of the PCR products from the 3 reactions of *18s* and *Pai-1*.

9.3 – Optimisation of PCR conditions

The induction of monocyte *Pai-1* by activated platelets was initially investigated using semi-quantitative RT-PCR. In terms of quantification of *Pai-1* mRNA, this technique was not appropriate due to its low sensitivity. To increase the sensitivity of the PCR reaction, real-time PCR was used. Two types of detection methods are available, and the first examined was SYBR-Green I. Upon binding to double stranded DNA the SYBR-Green I dye produces a fluorescence to allow detection. In comparison to ethidium bromide used in semi-quantitative RT-PCR, SYBR-Green I has a higher sensitivity, and better signal-to-noise ratio. After optimisation of parameters such as design of primers and template cDNA concentration, it was demonstrated that the SYBR-Green I method may still lack sensitivity for *Pai-1* mRNA in stimulated monocytes, and the *Pai-1* PCR sometimes produced non-specific products evident in the additional bands seen when the PCR products were visualised on an agarose gel. To address this problem, TaqMan was employed for the detection of PCR product. The sensitivity of detection was increased further as the 5' nuclease activity is based on the specific hybridisation of a dual labelled TaqMan. When the resulting PCR products were examined on an agarose gel, no additional bands were observed other than the product band, indicating no primer-dimer or non-specific product was yielded. In summary semi-quantitative PCR, although a cheaper method, required the most optimisation, and was the least specific and sensitive method. The sensitivity of detection with SYBR-Green I may be compromised by the formation of primers-dimers, lack of specificity and the formation of secondary structures in the PCR products. In the experiments discussed in this study,

real-time PCR (TaqMan) will be utilised to measure *Pai-1* due to its low level of expression.

The internal gene expression control used in these experiments was *18s*. Some have criticised the use of *18s* as a house-keeping gene due to the gene containing only one exon, therefore contamination of DNA would not be detected. There are a host of alternative control genes that could have been employed such actin and GAPDH. However, as mRNA was extracted using oligo-dT beads and the buffer contains DNAase, DNA contamination was not an issue under these experimental conditions. The expression of cyclophilin was also measured to validate the use of *18s* as a control gene (data not shown). There was no detectable difference between the expression of various monocyte preparations in *18s* and *cyclophilin* expression.