THE ROLE OF PROTEASE-ACTIVATED RECEPTOR-1 IN THE HUMAN VASCULATURE

BY

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ABSTRACT

Background Thrombin is a powerful cardiovascular agonist and a vital link between thrombosis and inflammation. In addition to its role in the coagulation cascade, it directly activates platelets, inflammatory cells, endothelium and vascular smooth muscle. Protease-activated receptor-1 (PAR-1) has been proposed as the principal thrombin receptor in man although its actions *in vivo* have not been defined. The aim of this thesis was to determine the direct vascular actions of PAR-1 agonism in the human venous and arterial circulations.

Objectives The effects of PAR-1 activation on dorsal hand vein diameter were measured by the Aellig technique in healthy volunteers, compared with activation of the trypsin receptor PAR-2, and further assessed in the presence or absence of norepinephrine, the glycoprotein (GP)IIb/IIIa antagonist tirofiban, and endothelial denudation. In the arterial circulation, forearm blood flow was measured by venous occlusion plethysmography. Intra-arterial PAR-1 activating peptide was co-infused with tirofiban, and compared with PAR-2 activation and bradykinin infusion. Platelet-monocyte binding (a sensitive measure of platelet activation) and tissue plasminogen activator release (t-PA) were measured throughout. In subsequent studies, effects of inhibition of the endothelium-dependent vasodilators nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarisation factor (EDHF) on PAR-1 activation were assessed, as well as comparing the effects of PAR-1 activation in smokers and non-smokers.

Methods Activation of PAR-1 caused dose-dependent venoconstriction ($P<0.001$) that was unaffected by norepinephrine or tirofiban co-infusion and endothelial denudation. In forearm resistance vessels, arterial PAR-1 activation increased forearm blood flow ($P<0.001$), t-PA release ($P<0.001$) and platelet-monocyte binding ($P<0.0001$). Activation of PAR-2 caused venous ($P<0.001$) and arterial ($P<0.01$) dilatation without t-PA release or platelet activation. Although blockade of prostacyclin production had no effect, PAR-1 induced arterial vasodilatation was attenuated by inhibition of NO synthesis ($P<0.0001$) and EDHF ($P<0.05$), and abolished by their combination ($P<0.01$). Smokers had impaired PAR-1 mediated vasodilatation and t-PA release.

Results We have, for the first time, demonstrated that PAR-1 agonism *in vivo* in man causes arterial dilatation, venoconstriction, platelet activation and t-PA release that is mediated through endothelium-dependent and independent pathways, and impaired in smokers. These unique and contrasting effects are of major physiological relevance to the regulation and resolution of intravascular thrombosis. These findings have implications for the development and therapeutic use of thrombin receptor antagonists and direct thrombin inhibitors.

To Kjartan and Agnar

CONTENTS

ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
Arg	Arginine
BD	Becton Dickinson
Ca^{++}	Calcium ions
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic 3'5'-guanosine monophosphate
EDHF	Endothelium-derived hyperpolarising factor
EET	Epoxyeicosatrienoic acid
eNOS	Endothelial nitric oxide synthase
FITC	Fluorscein isothiocyanate
GP	Glycoprotein
GPIs	Glycoprotein IIb/IIIa inhibitors
GTP	Guanosine 5'-triphosphate
INR	International Normalised Ratio
L-NMMA	L-N^G-monomethyl arginine citrate
LVDT	Linear variable differential transducer
MAP kinase	Mitogen-activated protein kinase
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor type 1
PAR-APs	PAR-activating peptides

PARs	Protease-activated receptors
PE	Phycoerythrin
PI3K	Phosphoinositide-3-kinase
PKC	Protein kinase C
PLCß	Phospholipase C beta
PPACK	D-phenylalanyl-L-propyl-L-arginine chloromethylketone
SEM	Standard error of the mean
Ser	Serine
SIGN	Scottish Intercollegiate Guidelines Network
SNP	Sodium nitroprusside
TEA	Tetraethylammonium ion
Thr	Threonine
t-PA	Tissue plasminogen activator
u-PA	Urokinase plasminogen activator
Val	Valine
vWF	von Willebrand factor

DECLARATION

In this thesis I present research that I undertook between February 2004 and January 2007, whilst working full-time as a British Heart Foundation Junior Research Fellow (FS 05/028) at the Centre of Cardiovascular Sciences, University of Edinburgh.

I was responsible for all stages of this project. As well as planning and writing all study protocols and grant and ethic applications, I was the principal investigator and carried out all the platelet and venous studies and a large majority of the arterial studies, analysed the findings and wrote up the results. Dr Ninian Lang carried out forearm studies on 8 volunteers in Chapter 5, and 12 volunteers in Chapter 6. In keeping with the nature of these studies, assistance with laboratory assays and vascular studies was received and acknowledged as appropriate.

All the work presented has been published in peer reviewed journals and we have copyright permission for inclusion of my published papers within this thesis.

The thesis has not been accepted in any previous applications for a degree and all sources of information have been acknowledged.

All studies were undertaken with the approval of and in accordance with the regulations of the Lothian Research Ethics committee and with the Declaration of Helsinki of the World Medical Association. Written informed consent was obtained from all subjects prior to their participation in the studies.

Ingibjörg J Guðmundsdóttir

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Professor David J Webb provided helpful advice regarding hand veins studies and further advice was received from Professor Ian Megson regarding platelet studies and from Professor Christopher Ludlam for laboratory assays. I want to thank my fellow researchers in the Cardiovascular Research group for great morale and team spirit. Nursing staff at the Cardiovascular Research Facilities at the Royal Infirmary of Edinburgh and Western General Hospital were essential in carrying out all the arterial studies. I would particularly like to acknowledge Pamela Dawson for all her work in carrying out the laboratory assays. I warmly thank Jean Cunningham with her help and encouragement on the final preparation of this thesis. This work would of course not have been possible without all my volunteers and I am indebted to them.

I am very grateful to the British Heart Foundation for enabling my to do this research with a Junior Research Fellowship grant (FS 05/028), as well as to the British Medical Association for a Lansdell and Lawson grant, and for a small project grant from the NHS Research and Development Fund.

Finally I want to thank my family for all their support and understanding: my parents, my partner Kristjan and our sons Kjartan and Agnar.

CHAPTER 1

INTRODUCTION:

THROMBIN RECEPTORS, PLATELET ACTIVATION, VASCULAR FUNCTION AND ENDOGENOUS FIBRINOLYSIS

1.1 THROMBIN

1.1.1 THROMBIN IN THE COAGULATION CASCADE

Blood coagulation prevents blood loss and has been crucial to survival throughout evolution. It is initiated by 'intrinsic' factors in the blood coming into contact with a foreign surface, such as collagen or subendothelial matrix, or in response to 'extrinsic' factors released by injured tissues, such as tissue factor. These intrinsic and extrinsic pathways of the coagulation cascade converge and both lead to thrombin activation. Thrombin (factor IIa) is mainly generated on the surface of platelets, from prothrombin synthesised in the liver. Thrombin plays an essential role in coagulation through its protease activity, leading to the formation of fibrin from fibrinogen, and generates the characteristic fibrin mesh of thrombus. Thrombus formation is further enhanced by positive feedback loops causing activation of factor V, VIII and X in the intrinsic pathway and factor VII in the extrinsic pathway. Factor XIII is also activated by thrombin and stimulates cross-linking and stabilisation of fibrin (Figure 1.1) [Patterson *et al*, 2001; Lane *et al*, 2005].

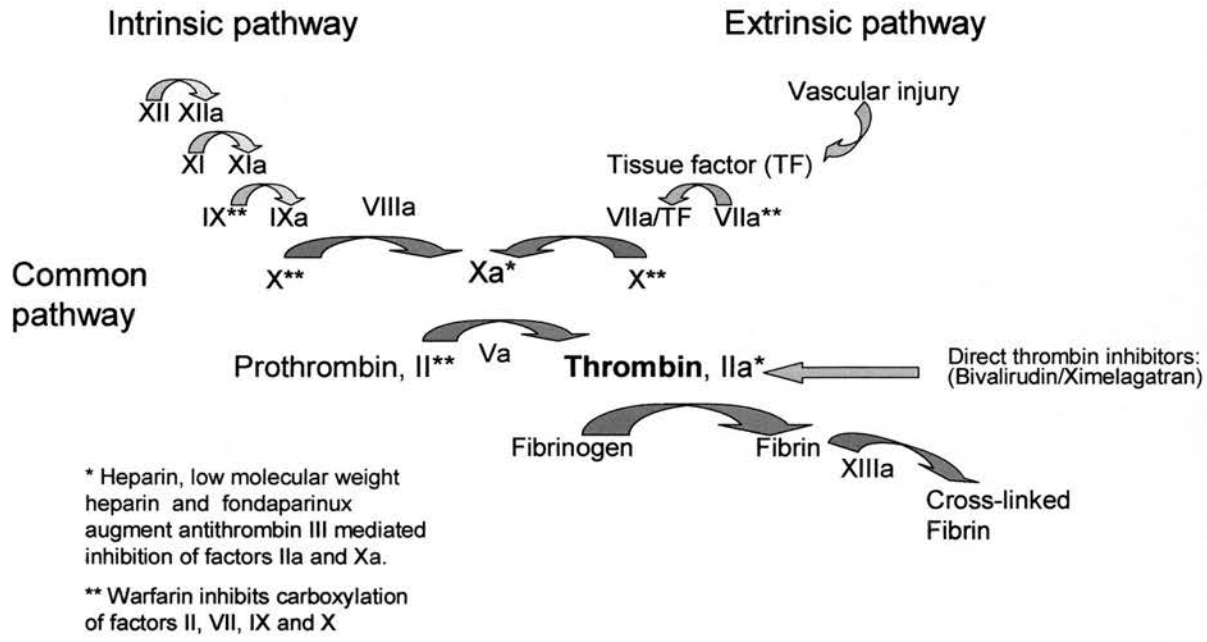

Figure 1.1 Schematic view of the coagulation cascade. TF - tissue factor.

1.1.2 RECEPTOR MEDIATED EFFECTS OF THROMBIN: PLATELETS AND ENDOTHELIUM

Apart from its central role in the coagulation cascade thrombin is one of the most important physiological agonists in the cardiovascular system and thrombin is at the interface between haemostasis, thrombosis and inflammation (Figure 1.2).

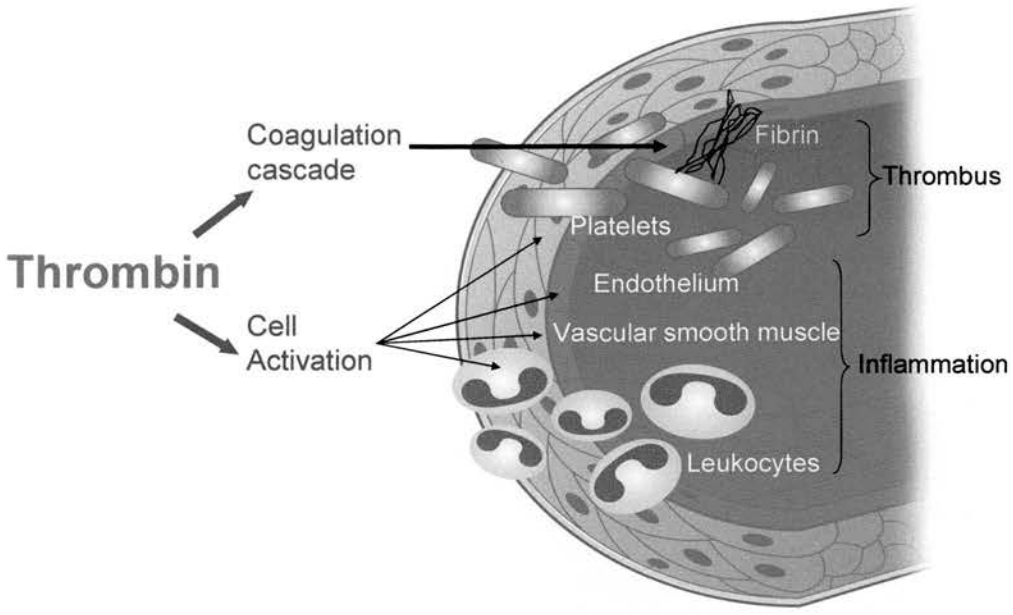

Figure 1.2 Thrombin stimulates fibrin production through its enzymatic activity in the coagulation cascade and causes cell activation through direct receptor mediated effects.

Thrombin causes platelet activation and aggregation, as well as activating leukocytes, endothelial and vascular smooth muscle cells. Thrombin also stimulates release of various cytokines and tissue-plasminogen activator (t-PA) [Patterson *et al*, 2001]. Thrombin is a vital link between thrombosis and inflammation, the key factors in wound healing and vascular injury as well as in pathophysiology such as in atherosclerosis and acute ischaemic events [Coughlin, 2005]. Identification of thrombin receptors has therefore been an important step in cardiovascular research.

1.2 PROTEASE-ACTIVATED RECEPTORS

1.2.1 CLASSIFICATION OF PROTEASE-ACTIVATED RECEPTORS

The apparent direct cellular effects of thrombin led to an extensive search for a thrombin receptor and eventually the discovery of a thrombin receptor. Once traditional ligand binding approaches proved fruitless, expression cloning methods were employed to conduct an extensive search for its receptor and used to identify functional human thrombin receptor cDNA [Rasmussen *et al*, 1991; Vu *et al*, 1991]. This led to the identification of G-protein coupled protease-activated receptors (PARs) that are characterised by a unique mechanism of activation: proteolytic cleavage unmasks a short peptide sequence that remains tethered and initiates transmembrane signalling via the receptor [Vu *et al*, 1991; Gerszten *et al*, 1994]. Four different types of PARs have been identified: PAR-1, -3 and -4 are all activated by thrombin. The type 1 receptor has been proposed as the principal thrombin receptor in man [Coughlin, 2000; Coughlin, 2005] and is widely expressed in many organ systems. Protease-activated receptor-1 is particularly important in human platelets, endothelial cells and vascular smooth muscle. Protease-activated receptor-1 is a high-affinity thrombin receptor, whereas PAR-4 requires a higher concentration of thrombin for activation of human platelets and PAR-4 does not appear to have important function in human endothelial cells [O'Brien *et al*, 2000]. The expression of PAR receptors differs between animals. In smaller animals PAR-4 is the main platelet receptor with PAR-3 mainly being a co-factor for activation of PAR-4 [Nakanishi-Matsui *et al*, 2000].

In contrast to PAR-1, -3 and -4, the PAR-2 receptor is activated by trypsin, tryptase and to a lesser extent, by coagulation factors upstream of thrombin [Hollenberg and Compton, 2002]. It appears to be of importance in inflammatory conditions that induce endothelial PAR-2 expression and vasodilatation [Ballerio *et al,* 2006].

1.2.2 PAR ACTIVATION AND PAR ACTIVATING PEPTIDES

Protease-activated receptors are G-protein coupled receptors that use a unique mechanism to convert proteolytic cleavage into a chain of diverse G-protein coupled signalling pathways. The protease cleaves the extracellular, amino-terminal of the receptor at a specific site that differs for each of the PARs, for thrombin the cleavage site on PAR-1 is between the amino acids arginine (Arg)[41] and serine (Ser)[42], and mutation of this cleavage site prevents thrombin-induced activation of the receptor [Vu *et al*, 1991]. This proteolytic cleavage exposes a new extracellular N-terminus of the receptor that remains tethered and binds to the body of the receptor to activate transmembrane signalling (Figure 1.3).

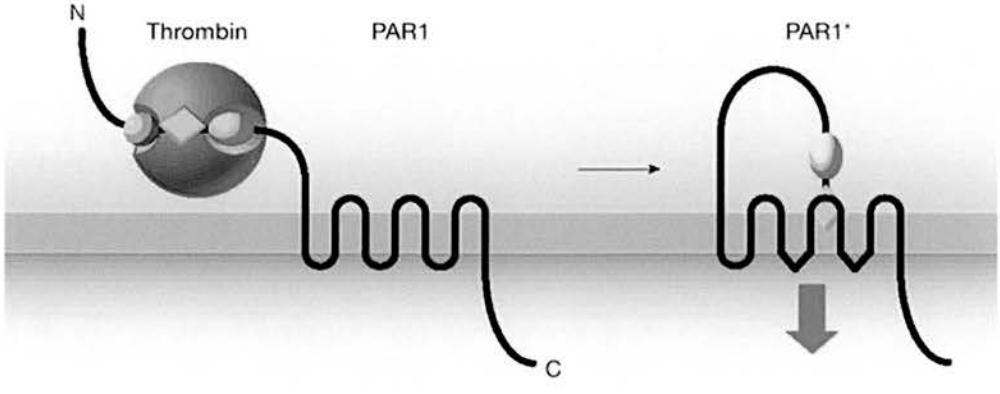

Figure 1.3 PAR activation: proteolytic cleavage, mainly by thrombin, exposes a new extracellular terminal of the receptor, that remains tethered and auto-activates the receptor [From Coughlin, 2000]. PAR-1 - protease-activated receptor-1.

By mimicking the newly exposed tethered ligand of the receptor, fully active synthetic peptides can be produced, that directly activate intact PARs without requirement for proteolytic cleavage of the receptor. Establishing the pharmacological structure of activating peptides for each of the PARs has opened avenues to studying the mechanism of receptor activation, the downstream G-protein coupled pathways and development of receptor antagonists. The development of selective PAR-1 agonists enables us to study the physiological role of the PAR receptors *in vivo*. In particular, the PAR-1 mediated effects of thrombin can be studied in isolation from its wider effects in the coagulation cascade whereas, it would not be possible to study the effects of thrombin itself in the human vasculature due to risk of vascular occlusion.

The characteristics of the extracellular ligand and corresponding protease-activated peptides have been studied in detail for each of the PARs [Hollenberg and Compton, 2002]. The protease-activating peptides can be as short as six amino acids in length and still retain full activity. For PAR-1, the endogenous tethered ligand domain is S^{42}FLLRNPNDKYEPF but the hexapeptide SFLLRN is a more potent agonist [Scarborough *et al*, 1992] and has been widely used in studies of the PAR-1 receptor. The endogenous sequence SFLLRN is also a weak agonist of PAR-2 receptor. However, replacing Ser^{1} with threonine $(Thr)^{1}$ generates TFLLRN which has similar potency to SFLLRN but is a more selective PAR-1 agonist [Hollenberg *et al*, 1997; Kawabata *et al*, 1999].

Protease-activated receptor PAR-2 activating peptides, derived from the human (SLIGKV) receptor sequences, can mimic the action of trypsin in selectively activating the PAR-2 receptor [Kawabata *et al*, 2004] and other activating peptides can be used to mimic the actions of thrombin on PAR-4. In contrast to PAR-1, PAR-2, and PAR-4, synthetic peptides corresponding to the tethered ligand of PAR-3 do not activate PAR-3. The reason for this is unknown. PAR-3 does not generate a signal when expressed alone, without other PARs, and appears to be mainly cross-activated via PAR-4 on mouse platelets but has little role in humans [Kahn *et al*, 1998].

1.2.3 TRANSMEMBRANE SIGNALLING

Activation of PAR receptors leads to coupling to G-proteins at the plasma membrane. Most of our understanding of thrombin-induced PARs signalling comes from extensive studies of PAR-1. Protease-activated receptor-1 can activate at least three distinctive G_α subunits: $G_{12/13}$, G_q and G_i to induce a wide array of downstream effects (Figure 1.4) [Hung *et al*, 1992; Offermanns *et al*, 1994; Barr *et al*, 1997]. Activation of G_q causes activation of phospholipase Cβ, elevation of cytoplasmic calcium and activation of protein kinase C with subsequent platelet degranulation and platelet aggregation [Offermanns *et al*, 1997b]. These pathways also cause stimulation of calcium regulated protein kinases and mitogen-activated protein (MAP) kinases which play a critical role in cell proliferation and differentiation. Stimulation of G_i inhibits adenylyl cyclase and reduces formation of cyclic adenosine monophosphate (cAMP) and stimulation of G_i also causes stimulation of phosphoinositide-3-kinase (PI3K), release of phosphatidylinositols and contributes to

platelet degranulation. Activation of $G_{12/13}$ induces platelet shape change via activation of Rho kinases [Klages *et al*, 1999], cellular migration and vascular genesis [Offermanns *et al*, 1997a]. These platelet activation mechanisms subsequently cause platelet aggregation by expression of the platelet integrin glycoprotein (GP)IIb/IIIa and GPIb and their cross-linking with von Willebrand factor (vWF) and fibrinogen [Davi and Patrono, 2007]. It is likely that different expression of these G-protein receptors explains how activation of the same receptor causes a diverse response in different tissues.

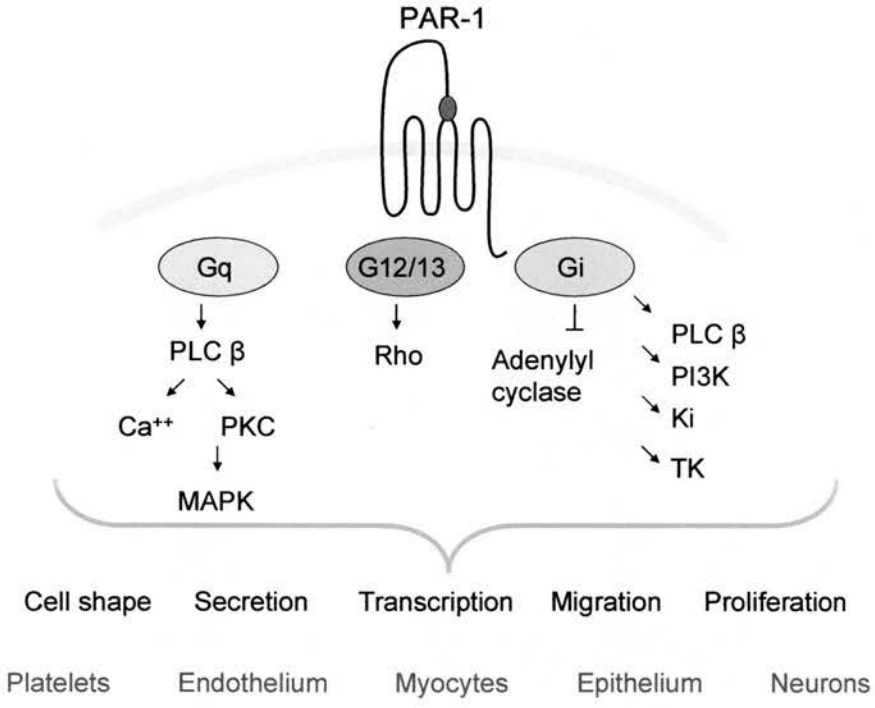

Figure 1.4 PAR-1 activation stimulates various G-protein coupled signalling pathways.
PAR-1 - protease-activated receptor-1; PLC β - phospholipase C beta; Ca^{++} - calcium ions; PKC - protein kinase C; MAPK - mitogen-activated protein kinase; PI3K - phosphoinositide-3-kinase; Ki - potassium ion channels; TK - tyrosine kinase.

Activation of PARs causes a transient signal, lasting a few minutes. Considering that PARs are activated by an irreversible mechanism, the question arises how the signal

is turned off. Protease-activated receptors-1 are rapidly internalised by endocytosis after activation [Trejo *et al*, 2000] and although a majority of the receptors undergo lysosomal degradation, a proportion is subsequently recycled to the cell surface [Shapiro *et al*, 1996]. A sustained signal would therefore require mobilisation of intact receptors from intracellular stores or the synthesis of new receptors. In endothelial cells and fibroblasts, prompt generation and mobilisation of PARs from internal stores is important. Platelets, however, neither have the ability to synthesise new receptors, nor have large intracellular stores, as thrombin causes irreversible platelet aggregation and platelets only need to respond once.

1.2.4 KNOCKOUT MICE AND ANIMAL STUDIES

Generation of genetically modified animals for PARs has been an important contribution for our understanding of their physiological function, in particular in the absence of widely available, selective antagonists. Knockout mice have been generated for each of the PARs [Connolly *et al*, 1996; Damiano *et al*, 1999; Lindner *et al*, 2000; Weiss *et al*, 2002]. These studies have demonstrated that approximately half of PAR-1 deficient mice (*PAR -/-* mice) die from bleeding at embryonic stage, whereas the surviving embryos appear to develop fairly normally to adult mice without bleeding diathesis [Connolly *et al*, 1996]. When endothelial development only is driven forward in the PAR-1 deficient embryos (by an endothelium specific promoter), the embryos develop normally suggesting that PAR-1 is required for normal angiogenesis and haemostasis in the mouse embryo [Griffin *et al*, 2001]. In mice, platelets of PAR-1 deficient animals remain responsive to thrombin. This and other studies demonstrated that PAR-1 receptors are not expressed or required for

activation of platelets in most rodents and small animals, but were subsequently found to express and be activated by PAR-4 and PAR-3. As PAR-1 is the most important thrombin receptor in human platelets, this difference in PAR expression markedly limits the use of small animals to further our understanding of PAR-1 in the human cardiovascular system.

1.2.5 PHYSIOLOGICAL ROLE OF PROTEASE-ACTIVATED RECEPTORS

Protease-activated receptors appear to be important in various organ systems. Their physiological role has not been fully established and depends in part on different expression of the various receptors between tissues.

PARs in the cardiovascular system

Given the imperative function of thrombin in the cardiovascular system, there has been a great interest in studying the physiological and pathophysiological role of PARs in the vasculature. The availability of specific PAR activating peptides has enabled us to study the receptor mediated effects of thrombin in separation from thrombin's effects in the coagulation cascade. Protease-activated receptors are expressed by endothelial and vascular smooth muscle cells, as well as by circulating blood cells. Extensive studies have been carried out *in vitro*, using vascular segments to study vasomotor effects and *ex vivo* platelet studies. Platelet studies have confirmed that PAR-1 and to a lesser extent PAR-4, but not PAR-2 or PAR-3, are the principal thrombin receptors on human platelets. Vasomotor studies have in general demonstrated that PAR-1, and to some extent PAR-2, appears to induce endothelium-dependent vasorelaxation [Kawabata *et al*, 2004] In mice, PAR-1

activation *in vivo* causes a transient hypotensive response, followed by a more sustained period of increase in blood pressure, whereas PAR-2 activation causes hypotension only [Cheung *et al*, 1998]. Activation of PAR-2 stimulates endothelial [Mirza *et al*, 1996] and vascular smooth muscle cell proliferation in cultures [McNamara *et al*, 1993] and induces angiogenesis in rodents [Milia *et al*, 2002]. The partial embryonic lethality caused by deletion of PAR-1 in knockout mice, is probably largely due to impaired angiogenesis [Griffin *et al*, 2001]. As mentioned above, due to species heterogeneity in PAR-1 expression, most rodent studies are, however, of limited relevance in humans. No previous studies have been done on the effects of PAR-1 receptor activation in the human vasculature *in vivo*, but a previous study looking at PAR-2 receptor activation has demonstrated that PAR-2 activation causes venous and arterial dilatation in humans [Robin *et al*, 2003].

PARs in other organ systems

The role of protease-activated receptors has been investigated extensively *in vitro* and in small animals and they have an important role in various tissues and organs. In the nervous system PARs are widely expressed, in particular PAR-1. This is likely to have implications for neuronal function, such as neurotransmission and pain, as well as during trauma and inflammation when low level stimulation may have a protective effect but high and prolonged stimulation may be detrimental [Vaughan *et al*, 1995; Luo *et al*, 2007]. In the gastrointestinal and respiratory tract PAR-2 is mainly involved in inflammatory conditions.

1.3 THROMBOSIS AND PLATELETS

1.3.1 THROMBOSIS: VENOUS AND ARTERIAL

Although blood coagulation prevents inappropriate haemorrhage and exsanguination following tissue trauma or injury, intravascular thrombosis is a major cause of morbidity and mortality. Arterial thrombosis causes tissue ischaemia including myocardial and cerebral infarction, and is the leading cause of death in western societies. Venous thrombosis can cause localised tissue oedema but is most serious when it embolises to the lung where it may cause life-threatening pulmonary thromboembolism.

Venous blood flow is slow and under low pressure. Thrombus formation is most common in the calf veins and mainly consists of red blood cells in a mesh of fibrin; so-called 'red thrombus'. Venous thrombosis is therefore readily prevented and treated by inhibiting thrombin-induced fibrin formation. Consistent with Virchow's triad, any condition that causes venous congestion (reduced flow), trauma (vessel wall damage) or activation of the coagulation system (hypercoagulability) will predispose to venous thrombosis. There are several well recognised risk factors including major surgery, malignancy (especially adenocarcinoma), immobility, genetic traits and oral contraceptives or hormone replacement therapy [Scottish Intercollegiate Guidelines Network (SIGN), 2002]. Most venous thrombi of the calves go unnoticed and small emboli frequently get filtered from the circulation in the lungs without causing symptoms. The risk of complications increases steeply if

the thrombus extends more proximally and detachment may lead to symptomatic pulmonary embolism. Venous thromboembolism prophylaxis, normally with low doses of heparin, is indicated in immobilised and post-operative patients. Longer treatment is indicated in those with confirmed proximal (above knee) deep vein thrombosis and pulmonary embolism but the length of treatment varies between 3 months and indefinitely [Scottish Intercollegiate Guidelines Network (SIGN), 1999; Hyers *et al*, 2001].

Arterial occlusion can be due to thrombosis *in situ*, such as with an acute coronary syndrome, or due to thromboembolism, such as a stroke from aortic or cardiac thrombus. Arterial thrombus tends to be caused by mechanical injury or plaque rupture of atherosclerotic arteries. The thrombus is rich in platelets connected by fibrin strands; so called 'white thrombus'. Agents that minimize either platelet activation or thrombin deposition are therefore used for prevention and treatment of arterial thrombus. In atrial fibrillation, cardiac thrombus forms because of atrial blood stagnation and is not as platelet rich [Kamath *et al*, 2001]. This may in part explain why aspirin is less effective in preventing stroke than warfarin in patients with atrial fibrillation [Petersen *et al*, 1989].

1.3.2 Platelets

Platelets are an essential part of thrombus formation stopping haemorrhage after vascular trauma. Platelet activation and aggregation is important in both the acute and chronic pathophysiology of atherothrombotic diseases where, as well as forming a haemostatic plug, they mediate inflammation. The discovery and extensive

employment of antiplatelet agents has been imperative to the advances in treatment and outcome of cardiovascular diseases such as myocardial infarction and stroke.

Platelets are produced by megakaryocytes and are anucleate cells. The initial tethering of platelets to the site of vascular injury is mediated via the vWF receptor complex GPIb/V/IX and subsequently strengthened through activation of integrins, in particular GPIIb/IIIa and GPIa/IIa. Following the initial tethering of the platelets further activation and augmentation of the response is mediated by several agonists, in particular thrombin, adenosine diphosphate (ADP), thromboxane A_2 and epinephrine, through G-protein coupled receptors [Offermanns 2006; Davi and Patrono, 2007]. Platelet activation causes further release of these mediators, shape change of the platelet cytoskeleton, release of platelet granules and expression of membrane proteins on the platelet surface and hence powerful positive feedback leading to platelet aggregation and formation of a platelet plug [Ruggeri, 2002]. Although physiologically necessary to ensure haemostasis at the site of vascular injury, platelets can also be activated by damaged endothelium in diseased arteries causing thrombotic events and tissue damage, in particular when release of substances that prevent platelet activation such as nitric oxide (NO), is hampered.

1.4 ENDOGENOUS FIBRINOLYSIS

The endogenous fibrinolytic system is responsible for the dissolution of arterial thrombi that are frequently found on the surface of atherosclerotic plaques at areas of

endothelial denudation [Davies *et al*, 1988; Oliver *et al*, 2005]. It is necessary for maintenance of vessel patency and prevention of tissue ischaemia.

Endogenous fibrinolysis involves clot dissolution where cross-linked fibrin undergoes hydrolytic cleavage by plasmin to generate fibrin degradation products. Fibrinolysis is regulated by the pro-fibrinolytic factor, tissue plasminogen activator (t-PA), and its endogenous inhibitor, plasminogen activator inhibitor type 1 (PAI-1).

1.4.1 PLASMIN AND PLASMINOGEN ACTIVATORS

Plasmin is a serine protease generated from plasminogen, a single chain glycoprotein consisting of 791 amino acids, principally produced by the liver [Raum *et al*, 1980]. Plasmin is formed by enzymatic cleavage of plasminogen at Arg^{561}-Valine $(Val)^{562}$ by plasminogen activators. Plasminogen activators are therefore required to initiate fibrinolysis. The two principal endogenous plasminogen activators are t-PA and urokinase plasminogen activator (u-PA). Whereas t-PA is the main plasminogen activator for intravascular fibrin degradation, u-PA appears more important for cell migration [Binder *et al*, 2007].

As well as regulating fibrinolysis, plasminogen activators appear to have other functions involving degradation of protein barriers, thereby facilitating cell migration and contributing to various physiological and pathophysiological processes such as embryogenesis, wound healing, angiogenesis and tumour growth and dissemination.

1.4.2 TISSUE PLASMINOGEN ACTIVATOR

Tissue plasminogen activator is a 527 amino acid glycoprotein, principally released from the endothelium [van Zonneveld *et al,* 1986a]. Under normal conditions total plasma t-PA concentration is around 70 pmol/L, and t-PA has a short half-life of about 5 minutes due to rapid hepatic clearance [Chandler *et al*, 1997]. Further t-PA can be rapidly released from small, dense endothelial storage vesicles [Eijnden-Schrauwen *et al*, 1995; Emeis *et al*, 1997] in response to various stimuli such as thrombin, bradykinin and substance P, as well as sympathetic stimuli such as exercise [Chandler *et al*, 1995; Newby *et al*, 1997b; Brown *et al*, 1999].

Tissue plasminogen activator alone is a weak plasminogen activator, but once bound to fibrin its catalytic activity rises 1000-fold due to conformational changes [van Zonneveld *et al*, 1986b]. The rapid mobilisation of t-PA from the endothelium is crucial, with thrombus dissolution being much more effective if t-PA is incorporated during, rather than after, thrombus formation [Fox *et al*, 1985]. Indeed, acute stimulated t-PA release predicts the future risk of cardiovascular events [Robinson *et al*, 2007].

1.4.3 PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1

Plasminogen activator inhbitor type 1 is the main t-PA inhibitor in man. It is a 379 amino acid glycoprotein that has a plasma concentration of around 500 pmol/L, and is produced by several tissues, mainly endothelial cells and vascular smooth muscle cells, but with a contribution from platelets and the liver [Simpson *et al*, 1991]. Plasminogen activator inhibitor type 1 is present in either an active or an inactive or

latent form in the circulation [Levin, 1995]. It is synthesised in the active form, which is unstable, with a half-life of approximately 30 minutes, although stability is enhanced by binding with vitronectin [Declerck *et al*, 1988; Seiffert *et al*, 1994]. The active form is converted to the latent form, which has a longer half-life of around 4 hours.

Plasma PAI-1 appears to be largely derived from endothelial and smooth muscle cells. Plasminogen activator inhibitor type 1 is also stored in high concentrations in the α-granules of platelets. During thrombotic events, when there are high levels of platelet activation, a large proportion of PAI-1 concentration can be derived from platelets [Brogren *et al*, 2004]. Platelet PAI-1 is, however, much less active than plasma PAI-1 derived from the endothelium (<5%), probably due to lack of the stabilising effects of vitronectin.

By binding to t-PA and thereby preventing t-PA binding to fibrin, a t-PA-PAI complex is formed which is eliminated from the circulation by the liver (Figure 1.5).

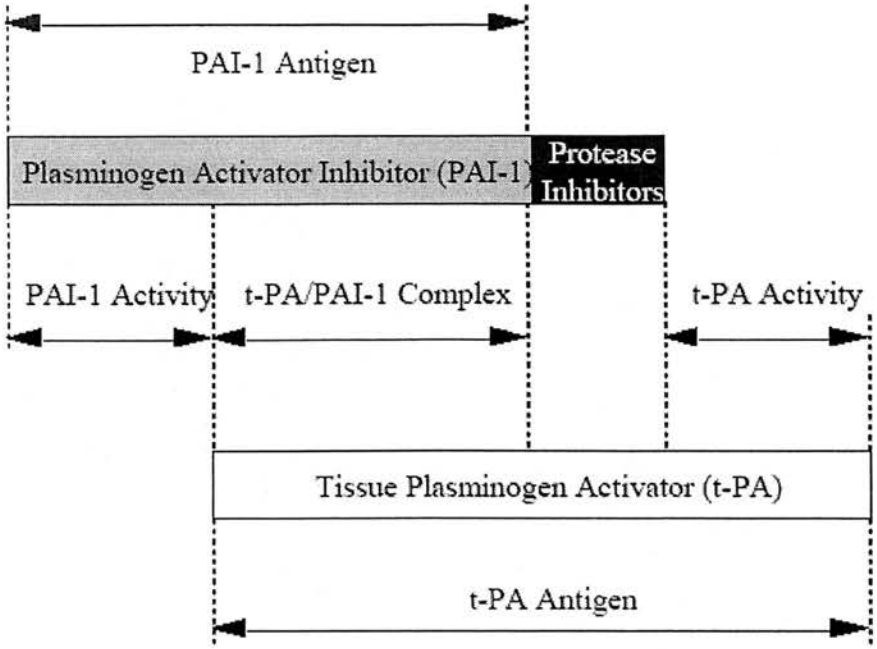

Figure 1.5 From Oliver *et al* [2005].

1.5 VASCULAR FUNCTION

1.5.1 VASCULAR ENDOTHELIUM

The endothelium is a vital organ, and not merely an inert physical barrier between blood and surrounding tissues. The endothelium is a single cell layer with a wide array of biological functions interacting with platelets, leukocytes and vascular smooth muscle cells to regulate vascular tone, haemostasis and fibrinolysis as well as releasing inflammatory mediators.

Regulation of vascular tone

The endothelium regulates blood flow by secretion of vasoactive compounds that either cause relaxation or constriction of blood vessels. A number of

endothelium-dependent agonists such as thrombin, bradykinin, acetylcholine and substance P have been identified that can act through three different mechanisms: NO-, prostacyclin- or endothelium-derived hyperpolarising factor (EDHF)-dependent pathways. The relative importance of each of these pathways differs between vascular beds and can be studied using pharmacological tools and knockout mice [Scotland *et al*, 2005].

Nitric oxide

Nitric oxide is the principal endothelium-derived relaxing factor. In their pioneering work, published in 1980 that subsequently led to their Nobel award, Furchgott and Zawadski first described the fundamental role of intact endothelium for acetylcholine mediated relaxation of blood vessels [Furchgott and Zawadski, 1980]. They proposed that the endothelium-dependent relaxation of arteries was mediated by an unstable substance, endothelium-derived relaxing factor. Two separate groups, led by Ignarro and Moncada, later simultaneously identified endothelium-derived relaxing factor as NO [Ignarro *et al*, 1987; Palmer *et al*, 1987]. Nitric oxide is formed by oxidation of L-arginine to L-citrulline by NO synthase in endothelial cells and platelets, or can be derived from an exogenous source i.e. NO donor drugs such as sodium nitroprusside. Nitric oxide diffuses into platelets and smooth muscle cells. The effects of NO are predominantly mediated via stimulation of soluble guanylate cyclase which catalyses synthesis of the secondary messenger cyclic 3'5'-guanosine monophosphate (cGMP) from guanosine 5'-triphosphate (GTP) [Lucas *et al*, 2000]. Cyclic GMP reduces platelet adhesion and aggregation via predominantly protein kinase C mediated pathways [Hofmann *et al*, 1992] and suppresses the binding of GPIIb/IIIa integrins to

fibrinogen as well as causing smooth muscle relaxation through reduction in intracellular calcium.

Endothelial nitric oxide synthase (eNOS) is constitutively expressed in endothelial cells, but up-regulated by various agonists including thrombin, bradykinin, substance P and acetylcholine. Flow mediated autoregulatory mechanisms in response to shear and mechanical stress, such as during ischaemia and hyperaemia, are also of importance [Loscalzo and Vita, 1994].

Prostacyclin

Prostacyclin is the second major endothelium-derived vasodilator and the predominant prostaglandin produced by endothelial cells. Prostacyclin is produced by cyclooxygenase from arachidonic acid, which is produced from membrane phospholipids by phospholipase A_2 [Smith, 1992]. Synthesis is stimulated by various agonists such as thrombin, bradykinin and adenosine nucleotides and can be inhibited by cyclooxygenase inhbitors. Prostacyclin has protective effects in the cardiovascular system. As well as being a vasodilator, it inhibits platelet activation and vascular smooth muscle cell proliferation. It also counteracts the prothrombotic effects of thromboxane A_2 released from platelets which causes vasoconstriction and platelet activation [Alfranca *et al*, 2006].

Endothelium-derived hyperpolarising factor

Endothelium-derived hyperpolarising factor refers to an unknown compound secreted by endothelial cells which leads to NO- and prostacyclin-independent

vasodilation by relaxation of vascular smooth muscle. This observed increased vasodilatation is inhibited by calcium-activated potassium channel blockers, and therefore relies on hyperpolarisation of vascular smooth muscle. The two main components of the EDHF-induced vasodilatation are the initial potassium channel-dependent endothelial hyperpolarisation and subsequent relay of the electrical signal through smooth muscle gap junctions. The exact chemical identity of EDHF remains elusive and good selective antagonists are lacking. Although no single compound has emerged as a universal EDHF, various substances may mediate endothelial relaxation via hyperpolarisation; for instance arachidonic acid derivates epoxyeicosatreionic acid (EET), hydrogen peroxide, potassium ions and C-type natriuretic peptide [Griffith, 2004].

Vasoconstricting factors

Vascular tone is maintained by the balance between vasodilators and vasoconstrictors. The best known vasoconstrictors are angiotensin II and endothelin-1. Endothelin-1, the most potent vasoconstrictor [Levin, 1995], is synthesised and released from the endothelium in response to hypoxia, shear stress and ischaemia and binds to the endothelin receptor on vascular smooth muscle cells leading to increased vascular tone. It augments the vasoconstrictor effects of catecholamines, as well as contributing to maintainance of basal vascular resistance [Davenport *et al*, 1990; Haynes *et al*, 1995]. Another well established and investigated vasoconstrictor is angiotensin II, released through the renin-angiotensin-aldosterone system: renin, released from the renal juxtaglomerular cells, converts angiotensinogen to angiotensin I. Angiotensin I is then converted to angiotensin II by

angiotensin-converting enzyme (ACE) which is mainly located on the surface of endothelial cells in the lungs. G-protein coupled angiotensin receptors subsequently cause increased blood pressure, vascular inflammation, production of aldosterone and various other cardiovascular responses [Peach, 1977; Brasier *et al*, 2002].

1.6 ANTITHROMBOTIC TREATMENT

1.6.1 ANTICOAGULATION

Anticoagulant therapy is used in the treatment and prevention of arterial and venous thrombosis in patients with a range of conditions including atrial fibrillation, mechanical prosthetic heart valves, deep venous thrombosis, acute coronary syndromes and pulmonary embolism. Current parenteral and oral anticoagulant therapies tend to act at multiple sites in the coagulation cascade and have significant drawbacks that limit their use. Warfarin and heparin are the most commonly used anticoagulant agents but have unpredictable pharmacokinetic and pharmacodynamic actions, potentially serious side-effects, and important drug and food interactions. They require regular repeated monitoring and dose adjustments. There is therefore a need for more convenient, predictable and reliable anticoagulant therapies.

Warfarin

Warfarin blocks the hepatic γ-carboxylation of the vitamin K-dependent clotting factors II, VII, IX and X. The onset of anticoagulant activity is delayed in accordance with the half-life of these factors: 2 to 3 days. Long-term anticoagulation with warfarin is considered in patients with atrial fibrillation, prosthetic heart valves, rheumatic heart disease, deep vein thrombosis or pulmonary embolism.

Treatment with warfarin is complicated by the need to assess liver function at onset and the continuous monitoring of anticoagulant control using the International Normalised Ratio (INR) of the prothrombin time. Achieving the appropriate level of anticoagulation is hindered by numerous and important drug-drug and drug-food interactions. Warfarin has a narrow therapeutic window and the risk of bleeding increases steeply with a small rise in INR. The risk of major haemorrhage (death, hospitalisation, intracranial haemorrhage or requiring blood transfusion) with warfarin is about 1-4% per year [Schulman, 2003]. Warfarin also inhibits production of the anticoagulant factors C and S. If their concentration falls dramatically, such as with excessive loading doses, or in people with hereditary Protein C or S deficiency, there is a temporary procoagulant state with the risk of small vessel thrombosis and skin necrosis. Warfarin can also be teratogenic and should be avoided in pregnancy.

Heparin

Heparin inhibits factors Xa and IIa (thrombin) by enhancing the activity of antithrombin III. Unfractionated heparin has largely been replaced by low molecular weight heparin for some indications, such as unstable angina and deep vein thrombosis, but remains a common anticoagulant in certain settings, such as in patients with acute renal failure, due to its short half-life.

The use of heparin can have several limitations and side-effects. Plasma proteins can bind to heparin and reduce its activity. Anticoagulation with unfractionated heparin is often unpredictable and requires frequent monitoring and dose titration.

Antithrombin III only works against free factor Xa and there is little activity against clot bound factor Xa. Heparin can cause platelet activation and one of the most serious non-haemorrhagic side-effects is heparin-induced thrombocytopenia. Heparin-induced thrombocytopenia is much less common with low molecular weight heparin and very unlikely with the new pentasaccharides [Amiral *et al*, 1997], such as fondaparinux that act indirectly to decrease thrombin generation by factor Xa inhibition.

Direct thrombin inhibitors

Direct thrombin inhibitors are a new class of anticoagulant therapy that act solely through direct enzymatic inhibition of thrombin. These drugs can either be administered orally or parenterally and inhibit both free and clot bound thrombin. Direct thrombin inhibitors also have antiplatelet effects [Nylander and Mattsson, 2003] and may augment thrombolysis induced by fibrinolytic agents [Visconte *et al*, 2004].

Oral direct thrombin inhibitors are the first oral anticoagulant agents since the introduction of warfarin over 60 years ago. There has been considerable interest surrounding the clinical use of these drugs as they do not require monitoring of INR, both for deep vein thrombosis prophylaxis after operations, and in patients with atrial fibrillation and after myocardial infarction. The first such drug, ximelagatran, was however withdrawn, mainly due to concerns regarding adverse effects on liver function. Deranged liver function tests, which are the most frequent side-effects (in about 6-7% of patients), are in most instances reversed back to normal either

spontaneously or after cessation of treatment. Evidence that ximelagatran could in very rare cases induce severe liver injury did however lead the American Food and Drug Administration to conclude that potential risk of side-effects might outweigh the benefit and that further studies were required. Another drug, dabigatran has subsequently been licensed for use in primary prevention of venous thromboembolic events after hip or knee replacement. Dabigatran does not appear to affect liver function as frequently and has now also been studied in patients with atrial fibrillation [Connolly *et al*, 2009].

Bivalirudin is a parenteral peptidic agent that has shown particular promise as anticoagulant treatment during percutaneous coronary intervention [Lincoff *et al*, 2003; Stone *et al*, 2007; Stone *et al*, 2008]. Bivalirudin is a recombinant polypeptide analogue of the carboxy-terminal of hirudin [Maraganore *et al*, 1990]. It is a direct thrombin inhibitor given by intravenous infusion and forms a bivalent complex with thrombin. The connection is slowly reversible due to proteolytic cleavage by thrombin [Witting *et al*, 1992] and its half-life is short at about 25 minutes [Fox *et al*, 1993]. In addition to its anticoagulant effects bivalirudin, unlike unfractionated heparin, causes additional inhibition of ADP-induced platelet aggregation in patients pretreated with clopidogrel who are undergoing percutaneous coronary intervention [Sibbing *et al*, 2008].

The effects of prolonged direct thrombin inhibition are unknown. Thrombin not only acts in the coagulation system but has a widespread physiological role in cell signalling, largely through activation of protease-activated receptors. Studies on

small animals have suggested that thrombin inhibition might reduce tumour invasion and metastases, and be beneficial in inflammatory conditions [Ossovskaya and Bunnett, 2004]. Long-term inhibition may furthermore cause up-regulation of the receptors and a rebound effect when thrombin receptor antagonists are withdrawn. We do however not have clinical studies on the long-term use of these medications in humans and it remains to be established what the balance will be between these potential benefits and possible adverse effects.

1.6.2 ANTIPLATELET TREATMENT

Acute atherothrombotic events such as myocardial infarction and stroke are the most common causes of death and morbidity in the western world. Antiplatelet agents are essential in the treatment of all cardiovascular diseases and have been a key element in advances made in this field in recent decades. The goal of antiplatelet treatment is to prevent platelet aggregation with minimal increase in the risk of bleeding.

Aspirin

Aspirin remains the most ubiquitously used and important antiplatelet agent and is established in the treatment of coronary, cerebrovascular and peripheral artery disease. Aspirin causes irreversible inhibition of platelet cyclooxygenase 1 (prostaglandin G/H synthase), suppressing the conversion of arachidonic acid to thromboxane A_2 [Patrono, 1994].

The acute and chronic use of aspirin in patients with atherothrombotic diseases has been extensively studied and consistently proven to be of great benefit in most

groups. The Antithrombotic Trialists' Collaboration meta-analysis [2002] established that aspirin treatment in patients with high risk of vascular disease reduces the incidence of death, myocardial infarction and stroke by 25%.

Clopidogrel

Aspirin alone is in many instances not sufficient to prevent platelet aggregation and the use of adjunctive antiplatelet treatment in addition to aspirin is increasingly common, in particular in patients at high risk of vascular events. Clopidogrel, a thienopyridine derivative, acts by disrupting ADP mediated platelet activation by binding to a platelet surface receptor P2Y12. Combination therapy of aspirin and clopidogrel has been shown to be valuable in patients with acute coronary syndromes and in patients undergoing percutaneous coronary intervention [Yusuf *et al*, 2001; Sabatine *et al*, 2005]. Long-term dual antiplatelet treatment with aspirin and clopidogrel in patients at high risk of atherothrombotic events does not have major benefits and is associated with increased risk of bleeding [Bhatt *et al*, 2006].

Glycoprotein IIb/IIIa inhibitors

All stimulants of platelets ultimately cause platelet aggregation via activation of the GPIIb/IIIa integrin complex and the subsequent bridging between platelets is further facilitated by fibrinogen and vWF. Glycoprotein IIb/IIIa inhibitors (GPIs) would therefore be expected to be the ultimate platelet antagonists. Glycoprotein IIb/IIIa inhibitors such as abciximab and tirofiban have been shown to be very efficient inhibitors of platelet aggregation when given intravenously in high risk patients undergoing percutaneous coronary intervention [Valgimigli *et al*, 2004;

Kastrati *et al*, 2006]. Results of trials of oral GPIs given for longer periods did however show adverse effects which may be due to lack of inhibition of platelet leucocyte binding and platelet microaggregation or possible pro-inflammatory effects [Storey *et al*, 1998; Nannizzi-Alaimo *et al*, 2003; Zhao *et al*, 2003].

Emerging antiplatelet agents

Despite combined antiplatelet treatment with currently available agents, platelet inhibition remains incomplete and acute thrombotic events continue to occur. It is therefore not surprising that this an active field for research and development of new therapeutic tools. Prasugrel and ticagrelor are emerging drugs that inhibit ADP-induced platelet activation [Jernberg *et al*, 2006; Wallentin *et al*, 2009], and thrombin-induced platelet aggregation may in the future be inhibited with the development of thrombin receptor antagonists (see below).

1.7 CLINICAL RISK FACTORS ASSOCIATED WITH ATHEROTHROMBOSIS

Cardiovascular diseases are becoming a leading cause of death worldwide, already being the most common cause of death in developed countries [Yusuf *et al*, 2004; Teo *et al*, 2006]. Large epidemiological studies, pioneered by the Framingham heart study, have helped to establish the most important clinical risk factors for atherothrombosis and this has had radical impact on our understanding, prevention and treatment of atherothrombotic diseases. Smoking, hypertension, hyperlipidaemia and diabetes are all well characterised clinical risk factors, but others such as lack of

exercise, obesity and depression also to play a role. Markers of inflammation such as highly sensitive c-reactive protein test can also be of value in predicting cardiovascular risk [Danesh *et al*, 2004].

1.7.1 SMOKERS

Smoking tobacco remains one of the most important and consistent modifiable risk factors for myocardial infarction and fatal coronary artery disease [Ambrose and Barua, 2004]. The INTERHEART study revealed that smoking tobacco increases the risk of non-fatal myocardial infarction by up to 7-fold [Teo *et al*, 2006]. The pathophysiological mechanisms underlying this association are likely to be a combination of accelerated atherosclerosis [Zieske *et al*, 2005] and a propensity to acute coronary thrombosis [Burke *et al*, 1997; Ambrose and Barua, 2004). Function of both circulating blood cells and endothelial cells is affected in smokers. Smokers have increased levels of platelet activation as measured by levels of platelet-moncyte binding [Harding *et al*, 2004]. Endothelium-dependent vasodilatation in both the peripheral and coronary circulation is impaired in patients with atherosclerosis [Ludmer *et al*, 1986]. Even subjects who do not have clinical evidence of cardiovascular diseases, but who have clinical risk factors such as smoking, have impaired endothelium-dependent vasodilatation and t-PA release in the peripheral and coronary circulations [Celermejer *et al*, 1996; Newby *et al*, 1999; Newby *et al*, 2001; Takashima *et al*, 2007] and this has predictive value for future cardiovascular events [Widlansky *et al*, 2003].

1.8 PROTEASE-ACTIVATED RECEPTORS IN DISEASE: PATHOPHYSIOLOGY AND THERAPEUTIC TARGETS

1.8.1 PATHOPHYSIOLOGICAL ROLE OF PARS

Protease-activated receptors are involved in the regulation of many physiological processes such as inflammation, coagulation, angiogenesis and neurotransmission. Their contribution to various diseases and possible therapeutic modulation is therefore intriguing. Inhibition of PAR-1 may in particular have beneficial antiplatelet effects [Derian *et al*, 2003] but further understanding of the effects of PAR-1 agonism and antagonism is required. Research and understanding of the role of PAR receptors in disease is however not straightforward. Expression of PARs differs between species and tissue types and is affected by disease processes such as inflammation and atherosclerosis. In the cardiovascular system, as well as being one of the principal receptors for platelet activation and aggregation, PARs contribute to vascular inflammation and restenosis [Andrade-Gordon *et al*, 2001].

PAR-1 and -2 are expressed and up-regulated in various tumour cells. Cancer metastasis relies on cell motility, degradation of basement membrane and stromal tissue and tumour angiogenesis. There is evidence that PAR-1 expression on cancer cells correlates with their invasiveness and metastatic potential [Even-Ram *et al*, 1998; Henrikson *et al*, 1999; Kaushal *et al*, 2006]. Protease-activated receptor-1 antagonists may therefore be useful as adjuvant treatment for some types of cancer.

1.8.2 PAR-1 ANTAGONISTS

Selective inhibition of PAR-1 reduces platelet activation and aggregation without affecting the enzymatic activation of fibrinogen in the coagulation cascade. Protease-activated receptor-1 antagonists have been used as a research tool for *in vitro* studies and their therapeutic role as antiplatelet agents is currently of major clinical interest.

Numerous PAR-1 antagonists (thrombin receptor antagonists) have been developed or are in development [Chackalamannil *et al*, 2003; Maryanoff *et al*, 2003; Nantermet *et al*, 2002; Seiler and Bernatowicz, 2003; Selnick *et al*, 2003] including the indazole based peptide mimetic RWJ-58259 (αS-N-[(1S)-3-amino-1-[[(phenylmethyl)-amino]carbonyl]propyl]-alpha-[[[[[1-(2,6-dichlorophenyl) methyl]-3-(1-pyrrolidinylmethyl)-1H-indazol-6-yl]amino]carbonyl]amino]-3,4-difluorobenzenepropanamide) [Andrade-Gordon *et al*, 2001; Damiano *et al*, 2003; Derian *et al*, 2003; Zhang *et al*, 2003] and a himbacine-derived thrombin receptor antagonist [Xia *et al*, 2007].

Data from a phase II clinical trial (see Chapter 7), where the use of the oral TRA SCH 530348 was compared with placebo in patients undergoing non-emergency percutaneous coronary intervention, in addition to other standard antiplatelet and antithrombotic treatment, have indicated that the use of thrombin receptor antagonists is safe and feasible [Becker *et al*, 2009].

1.9 HYPOTHESIS

Thrombin is one of the most important agonists in the cardiovascular system. Protease-activated receptors, in particular PAR-1, have been proposed as the principal thrombin receptors and PAR-1 antagonists are in clinical development as antiplatelet agents, but no data are available on the effects on PAR-1 activation *in vivo* in man.

In a series of clinical studies, the following hypotheses will be addressed:

1. PAR-1 activation causes concentration-dependent platelet aggregation and activation *in vitro*, and that PAR-1 induced platelet aggregation can be inhibited by specific PAR-1 antagonists.
2. PAR-1 activation causes venous dilatation *in vivo* in man.
3. PAR-1 activation induces arterial dilatation, platelet activation and acute release of endothelium dependent factors such as t-PA, *in vivo* in man.
4. PAR-1 mediated vasodilatation is endothelium-dependent, through nitric oxide, prostacyclin and endothelium-derived hyperpolarising factor release.
5. The effects of PAR-1 activation are altered in subjects with impaired endothelial function such as in smokers.

Currently available data stems from *in vitro* and animal studies but human data are lacking. After initial pilot studies looking at the effects of PAR activation on human platelet activation and aggregation *in vitro*, we intend to assess for the first time, the effects of PAR-1 activation in the venous and arterial circulation *in vivo* in man. Our

aim is to assess the effects of activation of this thrombin receptor on platelet function, release of endothelium-dependent factors and venous and arterial vasomotor function.

1.10 AIMS

The aims of the thesis were:

In platelets from healthy volunteers:

- To assess the effects of stimulation of PARs on human platelet activation as measured by levels of platelet-monocyte binding and on platelet aggregation.
- To assess the inhibitory effects of a specific PAR-1 antagonist, RWJ-58259, on platelet aggregation induced by the PAR-1 activating peptide SFLLRN, thrombin and by the thromboxane A_2 agonist U46619.
- To assess the effects of the glycoprotein IIb/IIIa inhibitor tirofiban on SFLLRN-induced platelet activation and aggregation.

In the venous circulation of healthy volunteers:

- To compare the effects of infusion of the PAR-1 activating peptide SFLLRN to infusion of the PAR-2 activating peptide SLIGKV in the dorsal hand vein.
- To assess the effects of SFLLRN infusion in the dorsal hand vein in the presence and absence of norepinephrine and tirofiban.

In the arterial circulation of healthy volunteers:

- To compare the effects of intra-arterial infusion of the PAR-1 activating peptide SFLLRN with the PAR-2 activating peptide SLIGKV and the endothelium-dependent vasodilator bradykinin on vasomotor function.
- To assess the effects of intra-arterial PAR-1 activation on release of t-PA, PAI-1 and vWF.
- To assess the effects of intra-arterial PAR-1 activation on platelet activation as measured by levels of platelet-monocyte binding.

To assess the role of endothelial function on the effects of PAR-1 activation in healthy volunteers:

- In the dorsal hand vein, to assess the effects of the PAR-1 activating peptide before and after endothelial denudation.
- In the forearm, assess the importance of endothelium-dependent pathways on PAR-1 induced vasomotor responses.

In smokers:

- To compare PAR-1 mediated vasomotor responses, and t-PA and PAI-1 release in the forearm circulation of cigarette smokers and healthy non-smoking control subjects.

CHAPTER 2

METHODS:

ASSESSMENT OF THE EFFECTS OF PROTEASE-ACTIVATED RECEPTOR STIMULATION ON PLATELETS, VASCULAR FUNCTION AND RELEASE OF ENDOTHELIUM-DERIVED FACTORS

2.1 INTRODUCTION

Cardiovascular events such as myocardial infarction and stroke are the result of a complex interaction between the endothelium, vascular smooth muscle, platelets, inflammatory cells and the coagulation cascade. Thrombin is a powerful physiological stimulator of inflammation and thrombosis and PARs, in particular PAR-1, are believed to be the principal thrombin receptors in man. Protease-activated receptors are widely expressed in humans in most tissues including the cardiovascular, respiratory and nervous systems and the gastrointestinal tract [Ossovskaya and Bunnett, 2004]. Previous studies of PAR-1 receptors have been carried out *in vitro* or in small animals but their role has not been previously studied *in vivo* man.

When studying a widely expressed receptor, such as PARs, systemic administration of a receptor agonist could cause concomitant effects in other organs such as the nervous system. This could potentially stimulate confounding neurohormonal reflexes, and vascular responses could thus not be solely attributed to the effects of direct vascular receptor stimulation [Webb, 1995]. Venous and arterial responses can, however, be studied directly without systemic influences, by assessing the effects of a localised infusion into the venous or arterial circulation with the Aellig dorsal hand vein technique and bilateral venous occlusion plethysmography [Aellig, 1981].

2.1.1 VENOUS STUDIES

By using the Aellig dorsal hand vein technique [Aellig, 1981], the direct effects of a locally active, subsystemic dose of infused drugs can be assessed in the venous circulation without systemic influences. As the doses required of the infused drugs are very small, and get rapidly diluted downstream from the dorsal hand vein, this is an excellent method to study drugs for the first time in man. Furthermore, dorsal hand veins are representative of the physiological regulation of central venous capacitance and cardiac preload, as cutaneous limb veins participate in sympathetic venomotor reflexes, unlike skeletal muscle veins [Benjamin *et al*, 1995].

2.1.2 ARTERIAL STUDIES

By using venous occlusion plethysmography, vascular responses can be directly assessed *in vivo* in response to unilateral brachial artery infusion of drugs at a subsystemic, locally active dose, where the infused arm is compared with the non-infused arm [Benjamin *et al*, 1995; Webb, 1995]. This method has been widely used and tested by our group and others. It has proven to be an excellent tool to assess vascular function and responses to various endogenous and exogenous factors in healthy subjects and patients with atherothrombotic diseases [Haynes *et al*, 1995; Newby *et al*, 1997a; Verhaar *et al*, 1998; Hrafnkelsdottir *et al*, 2004]. Furthermore, the antecubital fossae veins can be cannulated enabling intermittent blood sampling and measurements of endothelium released factors and inflammatory parameters.

2.2 STUDY SUBJECTS AND CONDITIONS

2.2.1 STUDY SUBJECTS

Study volunteers were healthy males and females, aged 20-38 years, who had not been taking any regular medications, including anti-inflammatory medications, over the counter medications, herbal supplements or vitamins. They did not have clinically significant co-existing conditions including hypertension, hyperlipidaemia, diabetes mellitus, asthma and coagulopathy, and had not suffered a recent infective or inflammatory condition. Study volunteers were not involved in other clinical trials at the time of the study.

2.2.2 ETHICS COMMITTEE APPROVAL AND INFORMED CONSENT

Each study was approved by the Lothian Research Ethics Committee, and conducted in accordance with the Declaration of Helsinki (1996) of the World Medical Association, and with the written informed consent of all volunteers.

2.2.3 STUDY CONDITIONS AND PREPARATION

All studies were carried out in a quiet, temperature controlled room (22-24°C). Participants were semi-recumbent (venous studies) or supine (arterial studies) and had abstained from alcohol for 24 hours, and from food and caffeine-containing drinks for at least 4 hours prior to the study.

2.3 DRUGS AND MATERIALS

For preliminary *in vitro* platelet studies, research grade PAR agonists were used. Protease-activated receptor-1 agonist SFLLRN, PAR-2 agonist SLIGKV, PAR-4 agonist GYPGQV and GPRP (to prevent thrombin-induced fibrin polymerisation in platelet rich plasma) were custom synthesised by Auspep, Parkville Victoria, Australia. RWJ-58259 was custom synthesised and molecular structure verified by mass spectral analysis and high performance liquid chromatography by American Peptide, Sunnyvale, California, USA. Lepirudin was obtained from Schering, Burgess Hill, UK. CD42a-fluorscein isothiocyanate (FITC), and control IgG1-FITC and CD14-phycoerythrin (PE) fluorochrome labelled monoclonal antibodies were obtained from Serotec Ltd, Oxford, UK and Dako UK Ltd, Buckinghamshire, UK, respectively. FACSLyse solution was from Becton Dickinson (BD), Oxford, UK. All other salts and reagents for *in vitro* studies were from Sigma, Gillingham, UK. Clinical grade compounds were used for all intravenous and intra-arterial infusions. Custom synthesised clinical grade SFLLRN and SLIGKV, as well as bradykinin and L-N^G-monomethyl arginine citrate (L-NMMA) and tetraethylammonium (TEA) were purchased from Clinalfa, Laufelfingen, Switzerland. Acetylcholine was from Novartis Pharmaceuticals UK Ltd, Frimley, UK, sodium nitroprusside (SNP) from David Bull Laboratories, Faulding, UK, norepinephrine from Abbott Laboratories, Maidenhead, UK, and tirofiban from Merck, Sharp and Dohme, Hoddesdon, UK.

2.4 PLATELET STUDIES

2.4.1 PLATELET AGGREGOMETRY

Blood was sampled with a 19-gauge needle from the antecubital fossa into a 50 mL syringe, transferred into tubes containing 3.8% citrate and centrifuged at 130 *g* for 20 minutes at room temperature to obtain platelet rich plasma. Blood was centrifuged at 1200 *g* for 10 minutes to obtain platelet poor plasma for reference samples. Platelet aggregation was measured at 37°C using standard optical platelet aggregometry [Sogo *et al*, 2000a; Crane *et al*, 2005; Guðmundsdóttir *et al*, 2005]. Platelet rich plasma and washed platelet samples were equilibrated at 37°C and stirred continuously. Aggregation was monitored in a four channel aggregometer (Chronolog 470 VS, Labmedics, Stockport, UK) linked to a MacLab 4s analogue-digital converter and Chart software (AD instruments, Sussex, UK) or PAP-4D, (Biodata Corporation, USA) linked to PC interface software (Platelet aggregation profiler® version 2.3, Biodata Corporation, USA). Aggregation was monitored for 5 minutes after addition of agonist alone or for 10 minutes after addition of agonist in the presence of antagonist.

Preparation of washed platelets

Washed platelets were prepared by centrifuging platelet rich plasma at 1200 *g* for 10 minutes in the presence of prostacyclin (300 ng/mL) and resuspending the platelet pellet in Hepes-Tyrodes buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM Hepes, 5 mM glucose; pH adjusted to 7.4), again with prostacyclin. This was centrifuged at 1200 *g* for 10 minutes and

resuspended in Tyrodes buffer solution with 1 mM Ca^{++} added. Hepes-Tyrodes buffer solution was used as reference control for washed platelets.

Preparation of RWJ-58259

Because of its highly lipophilic structure (logP of 6.1; Figure 2.1) [Damiano *et al*, 2003], the lyophilised powder of the PAR-1 antagonist RWJ-58259 [Damiano *et al*, 2003] was dissolved in DMSO to produce a 25 mM solution. Serial dilutions in 5% dextrose were used to obtain a 0.5 and 1 mM stock of RWJ-58259. The final concentration of DMSO in the platelet rich plasma/washed platelets was <0.5%.

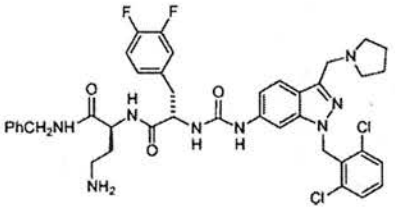

Figure 2.1 Structure of RWJ-58259.

Agonists

Preliminary *in vitro* platelet studies were carried out to construct concentration-response curves to define the concentration of the PAR-1 agonist SFLLRN that caused platelet activation and aggregation. SFLLRN (Auspep, Australia) was compared with thrombin (1 mM GPRP was added prior to thrombin to prevent fibrin

polymerisation in platelet rich plasma) and the thromboxane A_2 agonist U46619 (Sigma, UK) in both platelet rich plasma and washed platelets [Crane *et al*, 2005].

RWJ-58259

To assess the specificity of the PAR-1 activating peptide, both SFLLRN, thrombin and U46619-induced platelet aggregation were assessed in the presence and absence of the PAR-1 antagonist, RWJ-58259 (αS-N-[(1S)-3-amino-1-[[(phenylmethyl)-amino]carbonyl]propyl]-alpha-[[[[[1-(2,6-dichlorophenyl)methyl]-3-(1-pyrrolidinylmethyl)-1H-indazol-6-yl]amino]carbonyl]amino]-3,4-difluorobenzenepropanamide; American Peptide Company, USA) in washed platelets. Washed platelets were incubated with the antagonist for 2 minutes prior to addition of agonist (at EC_{50-70}, EC_{100} and supramaximal concentrations: thrombin 0.2, 0.3 and 1.0 U/mL; SFLLRN 1.8, 3.0 and 10.0 μM; and U46619 6.0 and 12 μM) and concentration response curves obtained.

Lepirudin

SFLLRN and thrombin-induced platelet aggregation was also assessed in the presence of the direct thrombin inhibitor, lepirudin (0.1 and 1.0 mg/mL; Schering, UK), a recombinant hirudin analogue [Gosselin *et al*, 2004].

Tirofiban

We chose the concentration of SFLLRN that caused near maximal platelet aggregation and assumed that the vascular and platelet PAR-1 receptor sensitivities were equivalent. However, before progressing to *in vivo* infusions, we defined the

dose of tirofiban that would completely inhibit platelet aggregation using the clinical grade preparation of SFLLRN. Platelet rich plasma was incubated for 5 minutes in the presence or absence of tirofiban (Merck, Sharp and Dohme, UK) at 50 ng/mL. Platelet aggregation was measured after the administration of 0.06-12 M of the clinical grade PAR-1 activating peptide SFLLRN (Clinalfa, Switzerland).

2.4.2 PLATELET-MONOCYTE BINDING

Five millilitres of venous blood were collected through a 19-gauge needle and transferred carefully into a tube containing the direct thrombin inhibitor, D-phenylalanyl-L-propyl-L-arginine chloromethylketone (PPACK). Five minutes after sampling, 60 µL of whole blood was incubated with monoclonal antibodies directly conjugated to fluorochromes for 20 minutes: FITC-conjugated IgG1, and PE-conjugated CD14 to label monocytes and FITC-conjugated CD42a with CD14-PE to label platelet-monocyte aggregates. The samples were then fixed with 500 µL FACSLyse solution (Becton Dickinson). Samples were run through a Beckman-Coulter XL2 flowcytometer. Monocytes and platelets were identified by gating for CD14 and CD42a positive cells respectively. Platelet-monocyte aggregates were defined as monocytes positive for CD42a. At least 3000 cells will be measured by flowcytometry and analyses performed using EXPO 32 software (Beckman-Coulter) software [Sarma *et al*, 2002].

Preliminary *in vitro* platelet studies were carried out to construct concentration-response curves to define the concentration of the PAR-1 agonist SFLLRN that caused platelet activation as measured by levels of platelet-monocyte binding. Blood

was collected into PPACK tubes with or without tirofiban (50 ng/mL). The PAR-1 agonist SFLLRN (0.06-6 μM) was added and whole blood incubated with monoclonal antibodies labelled with fluorochormes.

For *in vivo* studies of platelet-monocyte binding, whole blood was sampled simultaneously from the infused and non-infused arms at baseline and during infusion of study drugs, but no other agonists added and samples were processed as above.

2.5 DORSAL HAND VEIN STUDIES

2.5.1 DORSAL HAND VEIN INFUSION AND MEASUREMENT

A 23-gauge butterfly needle was sited in a dorsal hand vein, attached to a 16-gauge epidural catheter (Smiths Medical International, Watford, UK) and total infusion rate kept constant at 0.25 mL/min. If the subject returned for more than one study the same hand vein was used in all studies. The hand was supported above the level of the heart and an upper arm cuff inflated to 40 mmHg to obstruct venous return (Figure 2.2). The internal diameter of the dorsal hand vein was measured by the Aellig technique [Aellig, 1981]. In brief, a magnetised lightweight rod rested on the summit of the infused vein ≈1 cm downstream from the tip of the infusion needle. The rod passes through a core of a linear variable differential transformer (LVDT), supported above the hand by a small tripod. Changes in diameter of the vein cause vertical displacement of the rod, leading to a linear change in the voltage generated by the LVDT. This enables calculation of absolute changes in vein size.

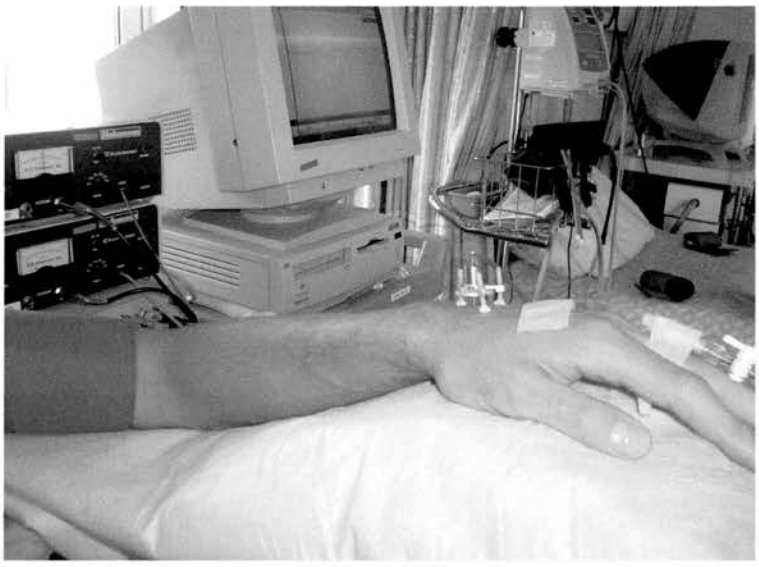

Figure 2.2 Aellig dorsal hand vein technique.

2.5.2 Effect of PAR activation on venous tone

Because dorsal hand veins do not have resting tone, norepinephrine (1-128 ng/min) was used to induce and maintain a 50-70% reduction in vein diameter throughout the study to allow detection of either venodilatation or venoconstriction. This was followed by co-infusion of the PAR-1 activating peptide SFLLRN (0.05-5 nmol/min) or the PAR-2 activating peptide SLIGKV (Clinalfa, Switzerland; 1.6-160 nmol/min).

2.5.3 Effect of glycoprotein IIb/IIIa receptor antagonism

The effects of SFLLRN (0.05-15 nmol/min) in the presence and absence of the GPIIb/IIIa inhibitor tirofiban (250 ng/min), and norepinephrine (1-128 ng/min) were assessed to determine the importance of platelet aggregation on PAR-1 mediated alterations of venous tone. The doses of tirofiban and SFLLRN were chosen to achieve end-organ concentrations equivalent to those with efficacy in *in vitro* studies and assumed a dorsal hand vein flow of 5 mL/min.

2.5.4 EFFECT OF ENDOTHELIAL DENUDATION

The effects of SFLLRN were assessed before and after localised endothelial denudation. First we established the presence of functional endothelium. As dorsal hand veins do not have resting tone, norepinephrine (1-128 ng/min) was used to induce 70% reduction in vein diameter. Once stable venoconstriction was obtained, acetylcholine (1 nmol/min; Novartis Pharmaceuticals UK Ltd, Frimley, UK) was co-infused with norepinephrine for 8 minutes to demonstrate endothelium-dependent venodilatation and an intact functional endothelium. Following a 20-minute saline infusion, the PAR-1 activating peptide, SFLLRN (0.05-15 nmol/min; Clinalfa, Switzerland), was administered intravenously before a final 20-minute saline washout infusion. At the end of the visit, the endothelium of the venous segment was denuded as previously described [Collier and Vallance 1989, Sogo *et al*, 2000b]. In brief, a second 23-gauge butterfly needle was sited 3-4 cm downstream from the tip of the infusion needle, and this segment of the vein isolated by the use of occlusion wedges. Distilled water was infused through the venous segment at a rate of 5 mL/min for 15 minutes, thereby causing endothelial denudation that persists for at least 2 days [Sogo *et* al, 2000b]. Aspirin (300 mg orally), was given 30 minutes prior to start of the first study, and on each of the two subsequent days to prevent venous thrombosis. On the second day following denudation, subjects re-attended and the first protocol was repeated using the same venous segment. In the absence of functional endothelium, acetylcholine co-infused with norepinephrine causes venoconstriction.

2.6 FOREARM VENOUS OCCLUSION PLETHYSMOGRAPHY

2.6.1 BRACHIAL ARTERY CANNULATION

The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd, Birmingham, UK) after infiltration of local anaesthesia with 1% lignocaine, and attached to a 16-gauge Portex epidural catheter (Smiths Medical International, Watford, UK). The total rate of infusion was kept constant throughout all studies at 1 mL/min (Figure 2.3).

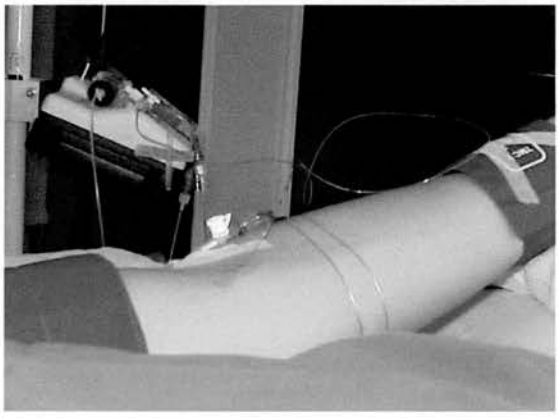

Figure 2.3 Brachial artery infusion.

2.6.2 FOREARM BLOOD FLOW

Forearm blood flow was measured in the infused and non-infused arms by venous occlusion plethysmography (Figure 2.4) using mercury-in-silastic strain gauges applied to the widest part of the forearm [Newby *et al*, 1999; Witherow *et al*, 2002]. During measurements the hands were excluded from the circulation by rapid inflation of the wrist cuffs to 200 mmHg for 3 minutes at a time. Upper arm cuffs were inflated intermittently to 40 mmHg to achieve venous occlusion and obtain

plethysmographic recordings (Figure 2.5). Supine heart rate and blood pressure were monitored at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer. Blood pressure was measured immediately after forearm blood flow measurements to avoid effects of venous congestion caused by blood pressure measurement on forearm blood flow.

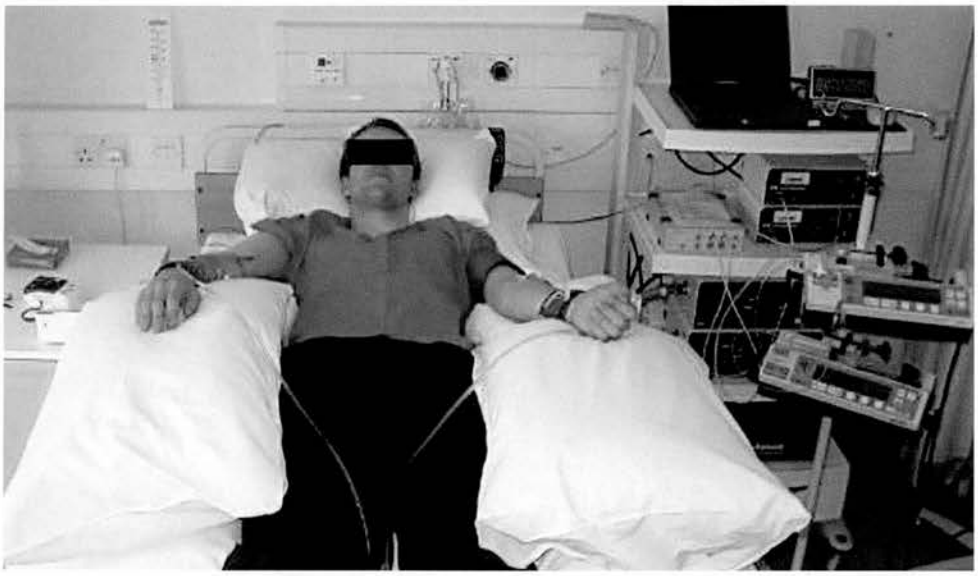

Figure 2.4 Forearm venous plethysmography.

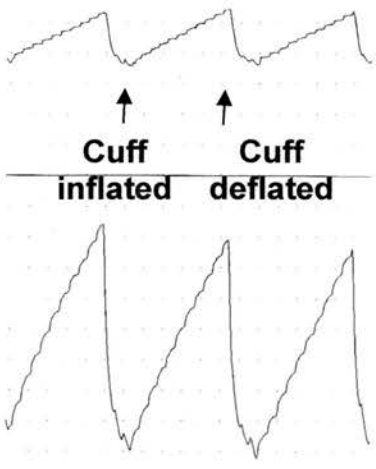

Figure 2.5 Forearm venous plethysmography recordings.

2.6.3 GPIIb/IIIa INHIBITION

Tirofiban (1.25 μg/min) was infused prior to and during PAR-1 activation to inhibit *in vivo* platelet aggregation and prevent potential thrombosis. The doses of tirofiban and SFLLRN were chosen to achieve end-organ concentrations equivalent to those with efficacy in *in vitro* studies and assumed a brachial artery blood flow of 25 mL/min.

2.6.4 PAR ACTIVATION

Forearm blood flow was measured by venous occlusion plethysmography in response to brachial artery infusion of PAR-1 activating peptide SFLLRN (5-50 nmol/min) with tirofiban (1.25 μg/min) and PAR-2 activating peptide SLIGKV (160-800 nmol/min) in eight healthy volunteers and compared with the effects of the endothelium-dependent vasodilator bradykinin.

2.6.5 INHIBITION OF NITRIC OXIDE AND PROSTACYCLIN SYNTHESIS

Forearm blood flow was measured by venous occlusion plethysmography in response to brachial artery infusion of SFLLRN (PAR-1 agonist; 5-50 nmol/min) with tirofiban (1.25 μg/min) in eight healthy volunteers on four visits using a randomised controlled cross-over study employing a two-by-two factorial design: with and without aspirin (600 mg orally; to inhibit prostacyclin synthesis) and the 'nitric oxide clamp'.

The 'nitric oxide clamp' was used to determine the contribution of NO in PAR-1 mediated vascular effects. Following baseline intra-arterial tirofiban infusion, the NO synthase inhibitor, L-NMMA (8 μmol/min), was co-infused. To compensate for L-NMMA induced basal vasoconstriction, forearm blood flow was returned to baseline using a titrated dose of exogenous NO in the form of intra-brachial sodium nitroprusside (90-900 ng/min). This dose of sodium nitroprusside was co-infused with L-NMMA and continued throughout the study. This arrangement allows a constant 'clamped' delivery of exogenous NO whilst endogenous NO synthase activity is inhibited.

2.7 BLOOD SAMPLING AND ASSAYS

Seventeen-gauge venous cannulae were inserted into left and right antecubital fossae. Blood samples were drawn simultaneously from each arm at baseline. Blood samples were also drawn before and after intra-arterial drug infusion. Blood was collected into acidified buffered citrate (Stabilyte, Biopool International, UK; for t-PA assays) and into citrate (Becton and Dickinson Vacutainer, BD UK Ltd, UK; for PAI-1, β-thromboglobulin and vWF assays). Samples were kept on ice before centrifugation at 2000 *g* for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma t-PA antigen and activity (t-PA Combi Actibind Elisa Kit; Technoclone, Austria), PAI-1 antigen and activity (Elitest-PAI-1 Antigen and Zymutest PAI-1 Activity; Hyphen Biomed, France), β-thromboglobulin (Asserachrom Btg, Diagnostica Stago, France) and vWF (Dako, Glostrup, Denmark)

concentrations were determined by enzyme-linked immunosorbant assays. Full blood count was measured at baseline and at the end of the studies.

2.8 DATA ANALYSIS AND STATISTICS

Dorsal hand venous [Haynes *et al*, 1995] and forearm plethysmographic [Newby *et al*, 1997a] data were analysed as described previously. Recordings for the first 60 seconds after wrist cuff inflation were not used because of variability in blood flow and reflex vasoconstriction this causes. In general the last five linear plethysmographic recordings in each 3-minute measurement period were used. Variables are reported as mean±standard error of the mean (SEM) and analysed using repeated measures, one- and two-way analysis of variance (ANOVA) with post-hoc Bonferroni corrections and two-tailed Students *t*-test as appropriate. Statistical analysis was performed with GraphPad Prism (Graph Pad Software) and statistical significance taken at the 5% level ($P<0.05$).

2.8.1 NET RELEASE OF ENDOTHELIUM-DERIVED FACTORS

The estimated net release of t-PA, PAI-1 and β-thromboglobulin antigen and activity was defined as the product of the infused forearm plasma flow (based on the mean haematocrit (Hct) and the infused forearm blood flow (FBF)) and the concentration difference between the infused ($[t\text{-}PA]_{inf}$) and non-infused ($[t\text{-}PA]_{non\text{-}inf}$) arms [Newby *et al*, 1997b] according to the formula:

$$\text{Estimated net t-PA release} = FBF \times (1\text{-}Hct) \times ([t\text{-}PA]_{inf} - [t\text{-}PA]_{non\text{-}inf})$$

CHAPTER 3

THE EFFECTS OF PROTEASE-ACTIVATED RECEPTOR STIMULATION AND INHIBITION ON PLATELET AGGREGATION AND ACTIVATION

3.1 SUMMARY

Inhibition of platelet activation has been central to advances in cardiovascular medicine over the last decades. Thrombin is one of the most important physiological stimulator of platelet activation. Protease-activated receptor-1 has been proposed as the principal thrombin receptor on human platelets and PAR-1 antagonists are in development as antiplatelet agents. The aims of this study were to study the effects of the PAR-1 activating peptide SFLLRN on platelet activation and aggregation and compare it with the effects of thrombin and the thromboxane A_2 agonist U46619. We have also assessed the inhibitory effects of the PAR-1 antagonist RWJ-58259 (αS-N-[(1S)-3-amino-1-[[(phenylmethyl)-amino]carbonyl]propyl]-alpha-[[[[[1-(2,6-dichlorophenyl)methyl]-3-(1-pyrrolidinylmethyl)-1H-indazol-6-yl]amino]carbonyl] amino]-3,4-difluorobenzenepropanamide), the direct thrombin inhibitor lepirudin, and those of the GPIIb/IIIa inhibitor tirofiban.

Thrombin, SFLLRN and U46619 caused concentration-dependent platelet aggregation (n=8). RWJ-58259 fully inhibited SFLLRN- (1.8 and 3 μM) induced aggregation (n=8, $P<0.0001$; one-way ANOVA; IC_{50}=2.1 μM). Thrombin (0.2-1 U/mL, EC_{70}–EC_{100}) mediated aggregation was only partially (up to 50%) inhibited by RWJ-58259 (n=8, $P<0.01$), but completely abolished by lepirudin (0.1-1 μg/mL; n=6, $P<0.001$). Tirofiban completely inhibited SFLLRN- ($P<0.001$; two-way ANOVA) induced platelet aggregation without affecting platelet activation (P=NS) as measured by levels of platelet-monocyte binding. We have, in preparation for subsequent *in vivo* studies, carried out this series of *in vitro* platelet studies and

demonstrated that the PAR-1 agonist SFLLRN causes concentration-dependent platelet activation and aggregation that can be selectively inhibited with the PAR-1 antagonist RWJ-58259. Thrombin-induced platelet aggregation is fully inhibited by the direct thrombin inhibitor lepirudin but only partially by RWJ-58259. We have furthermore demonstrated that PAR-1 induced platelet aggregation, but not platelet activation is inhibited by the GPIIb/IIIa inhibitor tirofiban.

3.2 INTRODUCTION

Inhibition of platelet activation has been pivotal to advances in cardiovascular medicine, primarily by inhibiting ADP and thromboxane A_2 mediated platelet activation. In spite of thrombin being recognised as one of the principal mediators of platelet activation, the identity of the thrombin receptor remained elusive until recently, and direct pharmacological inhibition was therefore previously not feasible.

Thrombin mediates thrombus formation through its central role in the coagulation cascade as well as having direct inflammatory and atherogenic effects. It is produced by the proteolytic activation of prothrombin through the final common pathway of the coagulation cascade, where it causes fibrin generation and cross-linking that underpins the basic structure of thrombus. In addition to its critical enzymatic role in blood coagulation, thrombin is one of the most important physiological stimulator of platelets, endothelial cells, vascular smooth muscle cells and leukocytes [Patterson *et al*, 2001]. An extensive search for a thrombin receptor led to the discovery of a group of G-protein coupled receptors termed protease-activated receptors

[Rasmussen *et al*, 1991; Vu *et al*, 1991] that mediate the cell signalling effects of thrombin [Coughlin, 2005]. Protease-activated receptors share a unique mechanism of activation where proteolytic cleavage of the receptor exposes a ligand that remains tethered and activates the receptor. To date, four different PARs have been identified, PAR-1, -3 and -4 are all activated by thrombin, whereas PAR-2 is mainly activated by trypsin [Hollenberg and Compton, 2002].

PAR-activating peptides (PAR-APs) have been designed to correspond to the tethered ligands of PARs and act as selective agonists [Hollenberg, 2000]. This makes it possible to study PAR mediated effects of thrombin in isolation from its enzymatic effects on fibrinogen and the coagulation cascade. Human platelets express both PAR-1 and PAR-4 [Chung *et al*, 2002), but not PAR-2 or PAR-3 [Ossovskaya and Bunnett, 2004], and thrombin mediates platelet activation in humans principally via PAR-1, as well as via PAR-4 and GPIb [Kahn *et al*, 1999; Lova *et al*, 2004]. Selective inhibition of PAR-1 could therefore have a therapeutic role by reducing platelet activation and aggregation without affecting the enzymatic activation of fibrinogen.

Numerous PAR-1 antagonists have been developed [Nantermet *et al*, 2002; Chackalamannil *et al*, 2003; Maryanoff *et al*, 2003; Seiler and Bernatowicz, 2003; Selnick *et al*, 2003] including the indazole based peptide mimetic RWJ-58259 (αS-N-[(1S)-3-amino-1-[[(phenylmethyl)-amino]carbonyl]propyl]-alpha-[[[[[1-(2,6-dichlorophenyl)methyl]-3-(1-pyrrolidinylmethyl)-1H-indazol-6-

yl]amino]carbonyl] amino]-3,4-difluorobenzenepropanamide) [Andrade-Gordon *et al*, 2001; Damiano *et al*, 2003; Derian *et al*, 2003a; Zhang *et al*, 2003].

The aims of this study were to assess the effects of PAR-1 and thrombin-induced platelet activation and aggregation *in vitro*, as well as the inhibitory effects of the specific PAR-1 antagonist RWJ-58259, the GPIIb/IIIa inhibitor tirofiban and the direct thrombin inhibitor lepirudin.

3.3 METHODS

3.3.1 SUBJECTS

Peripheral venous blood was drawn from healthy, non-smoking male and female volunteers (n=50). Participants had not been taking any regular medications, and did not have clinically significant co-existing conditions including hypertension, hyperlipidemia, diabetes mellitus, asthma and coagulopathy, and had not suffered a recent infective or inflammatory condition. The study was approved by the Lothian Research Ethics Committee, and conducted in accordance with the Declaration of Helsinki and with the written informed consent of all volunteers.

3.3.2 MATERIALS

Research grade PAR-1 agonist and GPRP were custom synthesised by Auspep, Australia. Clinical grade SFLLRN and SLIGKV were custom synthesised by Clinalfa, Switzerland. RWJ-58259 was custom synthesised and molecular structure verified by mass spectral analysis and high performance liquid chromatography by

American Peptide, USA. Lepirudin was obtained from Pharmion, UK. Tirofiban was obtained from Merck, Sharp and Dohme, UK. CD42a-FITC and control IgG1-FITC, and CD14-PE fluorochrome labelled monoclonal antibodies were obtained from Serotec Ltd, Oxford, UK and Dako UK Ltd, Buckinghamshire, UK, respectively. FACSLyse solution was from Becton Dickinson, Oxford, UK. All other salts and reagents were from Sigma, Gillingham, UK.

Preparation of RWJ-58259

Because of its highly lipophilic structure (logP of 6.1) [Damiano *et al*, 2003], the lyophilised powder of RWJ-58259 was dissolved in DMSO to produce a 25 mM solution. Serial dilutions in 5% dextrose were used to obtain a 0.5 and 1 mM stock of RWJ-58259. The final concentration of DMSO in the platelet rich plasma/washed platelets was <0.5%.

3.3.3 PLATELET AGGREGATION

Platelet preparation

Blood was collected from the antecubital fossa using a 19-gauge butterfly needle into a 50 mL syringe and transferred into tubes containing 3.8% sodium citrate. The blood was centrifuged at 130 *g* for 20 minutes at room temperature to obtain platelet rich plasma and washed platelets as previously described [Crane *et al*, 2005]. Platelet counts were adjusted to 200-350 x 10^9/L. Platelet rich plasma was used within 2 hours of sampling. Blood was centrifuged at 1200 *g* for 10 minutes to obtain platelet poor plasma for reference samples.

Washed platelets were prepared by centrifuging platelet rich plasma at 1200 *g* for 10 minutes in the presence of prostacyclin (300 ng/mL) and resuspending the platelet pellet in Hepes-Tyrodes buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM Hepes, 5 mM glucose; pH adjusted to 7.4), again with prostacyclin. This was centrifuged at 1200 *g* for 10 minutes and resuspended in Tyrodes buffer solution with 1 mM Ca^{++} added. Hepes-Tyrodes buffer solution was used as reference control for washed platelets.

Platelet aggregometry

Platelet aggregation was measured using standard optical platelet aggregometry [Sogo *et al*, 2000; Guðmundsdóttir *et al*, 2005]. Platelet rich plasma and washed platelet samples were equilibrated at 37°C and stirred continuously. Aggregation was monitored in a four channel aggregometer (Chronolog 470 VS, Labmedics, Stockport, UK) linked to a MacLab 4s analogue-digital converter and Chart software (AD instruments, Sussex, UK) or PAP-4D, Biodata Corporation, USA) linked to PC interface software (Platelet aggregation profiler® version 2.3, Biodata Corporation; USA). Aggregation was monitored for 5 minutes after addition of agonist alone or for 10 minutes after addition of agonist in the presence of antagonist.

Agonists

Concentration response curves were obtained in platelet rich plasma and washed platelets for thrombin (1 mM GPRP was added prior to thrombin to prevent fibrin polymerisation in platelet rich plasma), the PAR-1 agonist SFLLRN, the

thromboxane A_2 agonist U46619, as well as confirming that the PAR-2 agonist SLIGKV does not cause platelet aggregation.

PAR-1 antagonist, RWJ-58259

To assess response to the selective PAR-1 antagonist RWJ-58259, washed platelets were incubated with the antagonist for 2 minutes prior to addition of agonist (at EC_{50-70}, EC_{100} and supramaximal concentrations: thrombin 0.2, 0.3 and 1.0 U/mL; SFLLRN 1.8, 3.0 and 10.0 μM; and U46619 6.0 and 12 μM) and concentration response curves obtained.

Direct thrombin inhibitor, lepirudin

The effect of thrombin on platelet aggregation was tested in the presence of the direct thrombin inhibitor lepirudin, a recombinant hirudin analogue. Lepirudin (0.1 or 1 μg/mL [Gosselin *et al*, 2004] was added, with either 0.2 U/mL or 0.3 U/mL of thrombin.

Glycoprotein IIb/IIIa inhibitor, tirofiban

Before progressing to studies with SFLLRN infusions *in vivo*, we defined the dose of the GPIIb/IIIa inhibitor tirofiban that would completely inhibit platelet aggregation using the clinical grade preparation of SFLLRN. Platelet rich plasma was incubated for 5 minutes in the presence or absence of tirofiban at 50 ng/mL (Dickfeld *et al*, 2001; Judge *et al*, 2005]. Platelet aggregation was measured after administration of 0.06-12 μM SFLLRN.

3.3.4 PLATELET-MONOCYTE BINDING

Five millilitres of venous blood were collected through a 19-gauge needle and transferred into a tube containing the direct thrombin inhibitor, PPACK. For *in vitro* studies, blood was collected into two PPACK tubes with or without tirofiban (50 ng/mL) and incubated with clinical grade SFLLRN (0.06-6 μM). Five minutes after sampling, 60 μL of whole blood was incubated with monoclonal antibodies labelled with fluorochromes (IgG1-FITC and CD14-PE to label monocytes and CD14-PE/CD42a-FITC to label platelet-monocyte aggregates) for 20 minutes before the addition of 500 μL FACSLyse solution (Becton Dickinson). Samples were run through a Beckman-Coulter XL2 flowcytometer, and data analysis was performed using EXPO32 (Beckman-Coulter) software [Sarma *et al*, 2002].

3.3.5 STATISTICAL ANALYSIS

All results are expressed as a percentage of theoretical maximum platelet aggregation. Results were analysed using GraphPad Prism software and expressed as the mean±SEM. Analysis of variance was used to assess difference between groups. Statistical significance was assigned at $P<0.05$.

3.4 RESULTS

3.4.1 PLATELET AGGREGATION

Platelet aggregation to agonists

Thrombin, SFLLRN and U46619 caused concentration-dependent platelet aggregation (EC_{50}=1.26 μM for SFLLRN, 0.25 U/mL for thrombin, and 0.78 μM for U46619, Figure 3.1; $P<0.001$; one-way ANOVA, n=8).

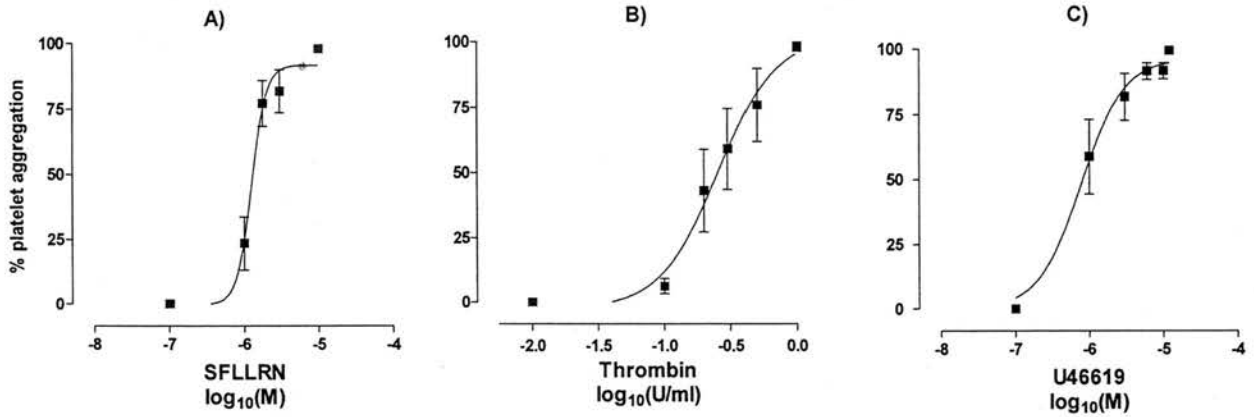

Figure 3.1 Induction of platelet aggregation in washed platelets 5 minutes after addition of A) the protease-activated receptor-1 (PAR-1) agonist SFLLRN, B) thrombin and C) the thromboxane A_2 agonist U46619. $P<0.001$; one-way ANOVA. All values are expressed as mean±SEM, n=8.
ANOVA - analysis of variance; SEM - standard error of the mean.

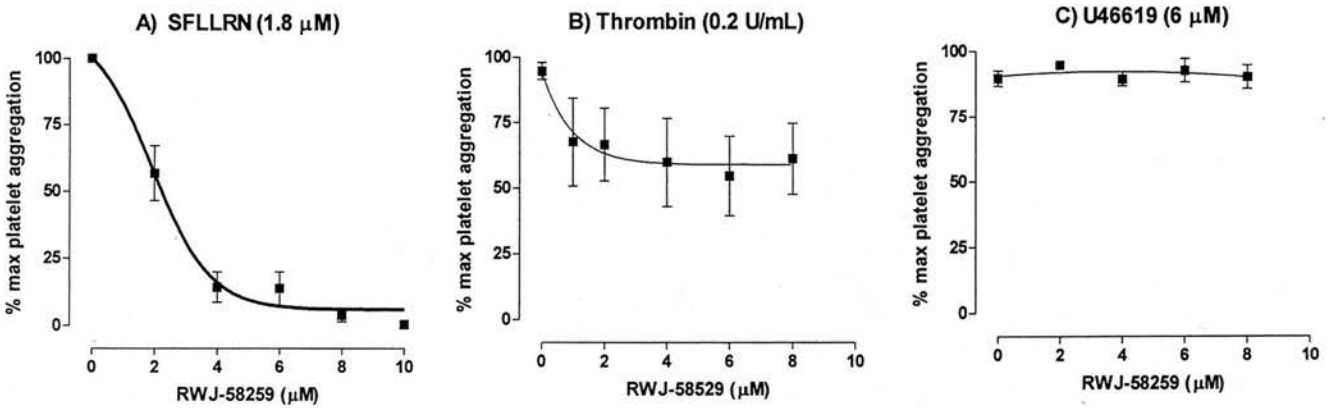

Figure 3.2 RWJ-58259 A) is a potent inhibitor of SFLLRN, $P<0.001$; B) partially inhibits thrombin, $P<0.01$; and C) does not affect U46619 mediated platelet aggregation in washed platelets. All values are expressed as mean±SEM, n=8 for all, one-way ANOVA.
SEM - standard error of the mean; ANOVA - analysis of variance.

Effects of PAR-1 antagonism

Protease-activated receptor-1 antagonism with RWJ-58259 caused a concentration-dependent reduction in aggregation and was able to inhibit completely platelet aggregation induced by 1.8×10^{-6} and 3×10^{-6} M SFLLRN (IC_{50}=2.1 μM; n=8, P<0.001; one-way ANOVA; Figure 3.2A), see sample platelet aggregometry tracing (Figure 3.3). In contrast, RWJ-58259 only partially inhibited thrombin-induced platelet aggregation (n=8, P=0.003 for 0.2 U/mL thrombin; one-way ANOVA; Figure 3.2B) and had no effect on platelet aggregation induced by the thromboxane A_2 agonist U46619 (n=8, P=NS; Figure 3.2C). Increasing the concentration of RWJ-58259 and thrombin did not affect the extent of the inhibition of platelet aggregation (P=NS).

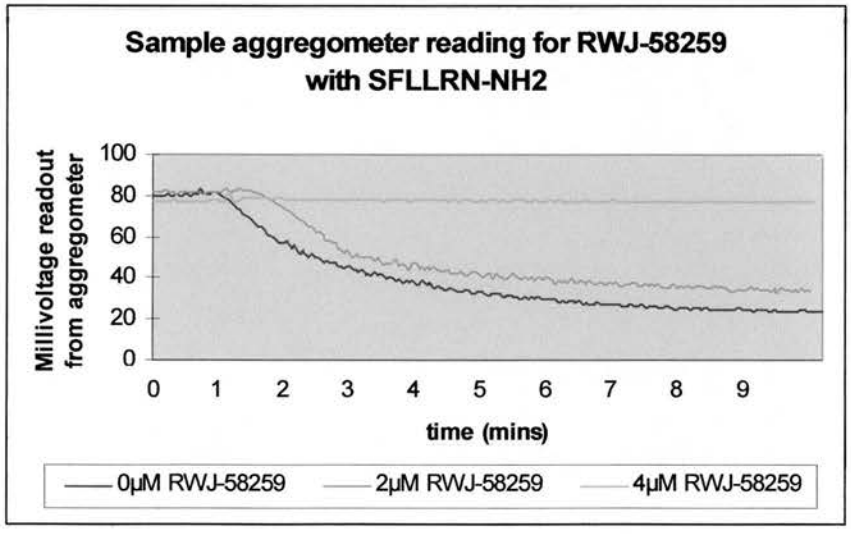

Figure 3.3. Sample platelet aggregometry tracing.

Direct thrombin inhibition

Lepirudin (1.0 and 0.1 mg/mL) completely inhibited thrombin (0.2 and 0.3 U/mL) mediated platelet aggregation (from 68±14% and 91±5% respectively to 0%, n=6,

P<0.001; ANOVA) without affecting SFLLRN mediated platelet aggregation (n=6, P=NS).

Glycoprotein IIb/IIIa inhibition

The clinical grade SFLLRN also caused a dose-dependent increase in platelet aggregation (EC_{50}=0.66 μM) that was inhibited by tirofiban (Figure 3.4A; n=6, P<0.001; two-way ANOVA).

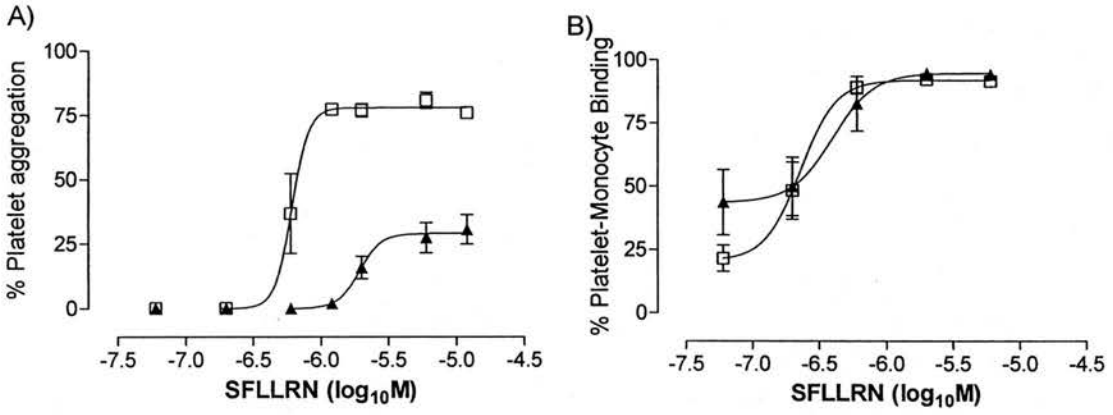

Figure 3.4 PAR-1 activating peptide (SFLLRN) induced (A) platelet aggregation and (B) platelet-monocyte binding in the presence (▲) or absence (□) of tirofiban (50 ng/mL). Mean±SEM. (A) P<0.0001; two-way ANOVA, *versus* tirofiban (B) P=NS; two-way ANOVA, *versus* tirofiban.
PAR-1 - protease-activated receptor-1; SEM - standard error of the mean; NS - non-significant; ANOVA - analysis of variance.

3.4.2 PLATELET-MONOCYTE BINDING

Protease-activated receptor-1 activation with clinical grade SFLLRN caused a dose-dependent increase in whole blood platelet-monocyte binding (EC_{50}=0.23 μM), that in contrast to platelet aggregation, was not affected by tirofiban (Figure 3.4B; n=6, P=NS; two-way ANOVA).

As anticipated, PAR-2 activation with SLIGKV induced neither platelet aggregation nor platelet-monocyte binding ($n \geq 3$; data not shown) at concentrations up to 3 mM.

3.5 DISCUSSION

Inhibition of platelet activation has been of major benefit in the prevention and treatment of cardiovascular diseases. Thrombin is one of the most potent physiological stimulants of platelet activation that appears to be mediated largely through the PAR-1 receptor although significant contributions are also provided by PAR-4 and GPIb receptors. To date, strategies to reduce the action of thrombin have focused on direct inhibition of its enzymatic activity. Recently, PAR-1 antagonists have been developed as potential antiplatelet agents [Chackalamannil *et al*, 2008] that do not affect the coagulation cascade. It is therefore important to define the *in vitro* and *in vivo* effects of PAR-1 agonism in man. As anticipated, we have demonstrated that PAR-1, but not PAR-2, agonism caused concentration-dependent platelet activation and aggregation. Moreover, in the presence of complete inhibition of platelet aggregation with the GPIIb/IIIa receptor antagonist tirofiban, PAR-1 agonism continued to induce platelet activation as measured by platelet-monocyte binding both *in vitro* and *in vivo*. This is consistent with previous findings that whilst GPIIb/IIIa receptor antagonism inhibits platelet aggregation, it does not affect platelet activation or platelet-monocyte binding [Sarma *et al*, 2002].

We have confirmed that the effects of the PAR-1 activating peptide SFLLRN are fully and selectively inhibited by the PAR-1 antagonist RWJ-58259. Whereas PAR-1

antagonism only partially inhibited the actions of thrombin, the direct thrombin inhibitor lepirudin abolished thrombin-induced platelet aggregation, consistent with its anticipated pharmacological effects.

The IC_{50} concentration of RWJ-58259 for PAR-1 agonist mediated platelet aggregation is similar to that reported by Damiano *et al* [2003], indicating the validity and efficacy of our preparation of this compound. Thrombin mediated aggregation was however only partly inhibited by RWJ-58259 and this was not augmented with increasing concentrations of the antagonist nor reduced by increasing concentrations of thrombin. This suggests that thrombin-induced platelet aggregation is, in part, mediated by non-PAR-1 mediated pathways.

Specific PAR-1 antagonists represent a potentially important novel and efficacious antithrombotic therapy in patients with cardiovascular diseases. Previous studies [Damiano *et al*, 2003; Derian *et al*, 2003a; Zhang *et al*, 2003] have indicated that RWJ-58259 is a potent inhibitor of both PAR-1 agonist and thrombin mediated platelet aggregation. However, there is limited specific information about the nature and extent of inhibition of thrombin mediated platelet aggregation by RWJ-58259 [Damiano *et al*, 2003; Derian *et al*, 2003a; Zhang *et al*, 2003]. A partial inhibitory effect on thrombin-induced platelet aggregation does not appear to be limited to RWJ-58259 with other PAR-1 antagonists including BMS-200261 [Bernatowicz *et al*, 1996] and SCH79797 [Ahn *et al*, 2000] demonstrating partial inhibitory actions on thrombin-induced aggregation [Chackalamannil *et al*, 2003].

Taken together, the findings with lepirudin and RWJ-58259, suggest the presence of an alternative platelet receptor for thrombin. PAR-4 receptors are present on human platelets and platelet aggregation can be induced with high concentrations (1 mM) of the human PAR-4 agonists GYPGQV [Andersen *et* al, 1999; Soslau *et al*, 2001]. In our studies, the PAR-4 agonist GYPGKV did not induce human platelet aggregation at concentrations up to 3 mM and PAR-4 activation is, in our opinion, unlikely to solely account for the PAR-1 independent platelet aggregation induced by thrombin. Other known PARs, PAR-2 and PAR-3 receptors are not expressed on human platelets [Ossovskaya and Bunnett, 2004]. A potential candidate is the GPIb receptor which is present on human platelets and can be activated by thrombin [Soslau *et al*, 2001].

In conclusion, we have demonstrated that the PAR-1 agonist SFLLRN causes concentration-dependent platelet activation and aggregation that can be selectively inhibited with the PAR-1 antagonist RWJ-58259. Thrombin-induced platelet aggregation is fully inhibited by the direct thrombin inhibitor lepirudin but only partially by RWJ-58259. We have furthermore demonstrated, in preparation for subsequent *in vivo* studies, that PAR-1 induced platelet aggregation, but not platelet activation can be inhibited by the glycoprotein IIb/IIIa inhibitor tirofiban.

CHAPTER 4

DIRECT VASCULAR EFFECTS OF PROTEASE-ACTIVATED RECEPTOR-1 AGONISM *IN VIVO* IN HUMANS

Guðmundsdóttir IJ, Megson L, Kell JS, Ludlam CA, Fox KAAA, Webb DJ, Newby DE.
Direct vascular effects of protease-activated receptor type 1 agonism *in vivo* in humans.
Circulation 2006;**114**:1625-1632.

4.1 SUMMARY

Protease-activated receptor-1 has been proposed as the principal thrombin receptor in man although its actions *in vivo* have not been defined. The aim of the study was to determine the direct vascular actions of PAR-1 agonism in man. Dorsal hand vein diameter was measured by the Aellig technique in fourteen healthy volunteers during local intravenous SFLLRN (PAR-1 agonist; 0.05-15 nmol/min) and SLIGKV (PAR-2 agonist; 1.6-160 nmol/min) infusions. The venous effects of SFLLRN were further assessed in the presence or absence of norepinephrine or the GPIIb/IIIa antagonist tirofiban in the arterial circulation. Forearm blood flow was measured by venous occlusion plethysmography in sixteen volunteers during infusion of SFLLRN (1-50 nmol/min), SLIGKV (160-800 nmol/min) and the endothelium-dependent vasodilator, bradykinin (100-1000 pmol/min). Platelet-monocyte binding (a sensitive measure of platelet activation), t-PA, PAI-1 and vWF concentrations were measured at intervals throughout the study.

Protease-activated receptor-1 activation with SFLLRN caused dose-dependent venoconstriction ($P<0.001$) that was unaffected by norepinephrine or tirofiban co-infusion. In forearm resistance vessels, SFLLRN increased forearm blood flow ($P<0.001$), t-PA release ($P<0.001$) and platelet-monocyte binding ($P<0.0001$) without affecting plasma PAI-1 or vWF concentrations. Protease-activated receptor-2 activation with SLIGKV caused venous ($P<0.001$) and arterial ($P<0.01$) dilatation without t-PA release.

We have, for the first time, demonstrated that PAR-1 agonism causes platelet activation, venous constriction, arterial dilatation and t-PA release *in vivo* in man. These unique and contrasting effects provide important insights into the physiological and pathophysiological role of thrombin in the human venous and arterial circulations.

4.2 INTRODUCTION

Thrombin is a powerful physiological stimulant in the cardiovascular system. Apart from its central enzymatic role in the coagulation cascade, it directly activates platelets, leukocytes, and vascular smooth muscle and endothelial cells [Patterson *et al*, 2001]. Thrombin is therefore a vital link between thrombosis, cellular activation and inflammation; key pathogenic factors in atherothrombotic disorders.

Given the apparent direct cellular effects of thrombin expression, cloning methods [Rasmussen *et al*, 1991; Vu *et al*, 1991] were employed to conduct an extensive search for its receptor. This led to the identification of G-protein coupled PARs that are characterised by a unique mechanism of activation whereby proteolytic cleavage unmasks a short peptide sequence that remains tethered and activates the receptor [Vu, 1991; Swift *et al*, 2006]. Four different types of PARs have been identified: PAR-1, -3 and -4 are all activated by thrombin. The type 1 receptor has been proposed as the principal thrombin receptor in man [Coughlin 2000; Coughlin, 2005]. In contrast, the PAR-2 receptor is activated by trypsin, tryptase and to a lesser extent by coagulation factors upstream of thrombin [Hollenberg and Compton,

2002]. It appears to be of importance in inflammatory conditions that induce endothelial PAR-2 expression and vasodilatation.

Protease-activated receptor-1 agonism has been extensively studied *in vitro* and is associated with platelet activation and aggregation [Chung *et al*, 2002], vasodilatation [Kawabata *et al*, 2004] and angiogenesis [Griffin *et al*, 2001]. Although studies in small animals suggest that PAR-1 and PAR-2 agonism induces vasodilatation [Damiano *et al*, 1996; Cheung *et al*, 1998], there is significant species heterogeneity and rodent models are of limited relevance to man [Kinlough-Rathbone *et al*, 1993]. Exploring the role of PAR-1 receptors in the human vasculature would deepen our understanding of the physiological role of thrombin and be of interest in the development of new therapeutic strategies, such as PAR-1 receptor antagonists [Damiano *et al*, 2003; Chackalamannil *et al*, 2005] and direct thrombin inhibitors [Ebrahimi *et al*, 2005; Serruys *et al*, 2006].

The aims of the present study were therefore to determine for the first time, the direct *in vivo* vascular effects of PAR-1 agonism in the human vasculature. Specifically, we wished to assess the direct role of PAR-1 activation on venous and arterial tone, platelet activation and the release of endothelium-derived factors *in vivo* in man and to compare them with the effects of PAR-2 activation.

4.3 METHODS

4.3.1 SUBJECTS

Thirty healthy non-smokers (aged 21-32 years) were recruited into the study (Table 4.1). Participants had not been taking any regular medications, over the counter medications, herbal supplements or vitamins. They did not have clinically significant co-existing conditions including hypertension, hyperlipidaemia, diabetes mellitus, asthma and coagulopathy, and had not suffered a recent infective or inflammatory condition. The study was approved by the Lothian Research Ethics Committee, and conducted in accordance with the Declaration of Helsinki and with the written informed consent of all volunteers.

TABLE 4.1 Baseline characteristics of study volunteers. Mean± SEM

Heart Rate (/min)	67±2
Blood Pressure (mmHg)	
Systolic	132±2
Diastolic	73±1
Haematocrit	0.42±0.01
White Cell Count (x 10^9/L)	4.98±0.37
Platelet Count (x 10^9/L)	213±11

SEM - standard error of the mean.

All studies were carried out in a quiet, temperature controlled room (22-24°C). Participants were semi-recumbent (venous studies) or supine (arterial studies) and

had abstained from alcohol for 24 hours, and from food and caffeine-containing drinks for at least 4 hours prior to the study.

4.3.2 DRUGS

SFLLRN, SLIGKV, bradykinin (Clinalfa, Laufelfingen, Switzerland), norepinephrine and tirofiban were all administered following dissolution in 0.9% saline. All solutions were freshly prepared on the day of the study.

4.3.3 VENOUS STUDIES

A 23-gauge butterfly needle was sited in a dorsal hand vein in the direction of flow and total infusion rate kept constant at 0.25 mL/min in all studies. The hand was supported above the level of the heart and an upper arm cuff inflated to 40 mmHg to obstruct venous return. The internal diameter of the dorsal hand vein was measured by the Aellig technique [Aellig, 1981]. A magnetised lightweight rod rested on the summit of the infused vein approximately 1 cm downstream from the tip of the infusion needle. The rod passes through a core of a LVDT, supported above the hand by a small tripod. Changes in diameter of the vein cause vertical displacement of the rod, leading to a linear change in the voltage generated by the LVDT. This enables calculation of absolute changes in vein size.

Protocol 1: Effect on venous tone

Because dorsal hand veins do not have resting tone, norepinephrine (1-128 ng/min) was used to induce and maintain a 50-70% reduction in vein diameter throughout the study to allow detection of either venodilatation or venoconstriction. This was

followed by co-infusion of the PAR-1 activating peptide SFLLRN (0.05-5 nmol/min) or the PAR-2 activating peptide SLIGKV (1.6-160 nmol/min).

Protocol 2: Effect of glycoprotein IIb/IIIa receptor antagonism

The effects of SFLLRN (0.05-15 nmol/min) in the presence and absence of the GPIIb/IIIa inhibitor, tirofiban (250 ng/min), and norepinephrine (1-128 ng/min) were assessed to determine the importance of platelet aggregation on PAR-1 mediated alterations of venous tone. The doses of tirofiban and SFLLRN were chosen to achieve end-organ concentrations equivalent to those with efficacy in *in vitro* studies (Chapter 3) and assumed a dorsal hand vein flow of 5 mL/min.

4.3.4 ARTERIAL STUDIES

All subjects underwent brachial artery cannulation with a 27-standard wire gauge steel needle under controlled conditions. Intra-arterial infusion rate was kept constant at 1 mL/min throughout all studies. Forearm blood flow was measured in the infused and non-infused arms by venous occlusion plethysmography using mercury-in-silastic strain gauges as described previously [Newby *et al*, 1997a; Newby *et al*, 1999]. Supine heart rate and blood pressure were monitored at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer. Tirofiban (1.25 μg/min) was co-infused during PAR-1 activation to inhibit *in vivo* potential platelet aggregation. The doses of tirofiban and SFLLRN were chosen to achieve end-organ concentrations equivalent to those with efficacy in *in vitro* studies using the clinical grade preparation of SFLLRN and the dose of tirofiban that completely inhibited platelet aggregation *in vitro* (Chapter 3) and assumed a brachial

artery blood flow of 25 mL/min. Pilot studies were also carried out starting with much lower intra-arterial doses (0.05 nmol/min) that produced no vascular effects.

Blood sampling

Seventeen-gauge venous cannulae were inserted bilaterally into the antecubital fossae. Blood samples were drawn simultaneously from each arm during infusion of saline, tirofiban and each dose of the PAR activating peptides and bradykinin. They were collected into acidified buffered citrate (Stabilyte, Biopool International, UK; for t-PA assays) and into citrate (Becton Dickinson Vacutainer, BD UK Ltd, UK; for PAI-1 and vWF assays). Samples were kept on ice before centrifugation at 2000 *g* for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma t-PA antigen and activity (t-PA Combi Actibind Elisa Kit; Technoclone, Austria) and PAI-1 antigen and activity (Elitest-PAI-1 Antigen and Zymutest PAI-1 Activity; Hyphen Biomed, France) concentrations were determined by enzyme-linked immunosorbant assays. Full blood count was measured at baseline and at the end of the study.

Platelet-monocyte binding

Blood was also collected from each arm for determination of levels of platelet-monocyte binding *in vivo*; at baseline, the highest dose of SFLLRN, during saline washout and at the highest dose of bradykinin. Five millilitres of venous blood were collected and transferred into a tube containing the direct thrombin inhibitor, PPACK. Five minutes after sampling, blood was incubated with appropriate monoclonal antibodies labelled with fluorochromes for 20 minutes and platelet-

monocyte aggregates measured as described previously in Chapter 3 [Harding *et al*, 2004].

Protocol 3: PAR-1 activation

Following a 20-minute baseline saline infusion, tirofiban (1.25 μg/min) was infused throughout the study. Thirty minutes from the commencement of the tirofiban infusion, the PAR-1 activating peptide SFLLRN was co-infused at 5, 15 and 50 nmol/min for 8 minutes at each dose separated by 6-minute saline washout infusions. This was followed by a 30-minute washout period before co-infusion of bradykinin (an endothelium-dependent vasodilator that releases t-PA; Clinalfa, Switzerland) at 100, 300, and 1000 pmol/min [Witherow *et al*, 2002] for 8 minutes at each dose, Figure 4.1.

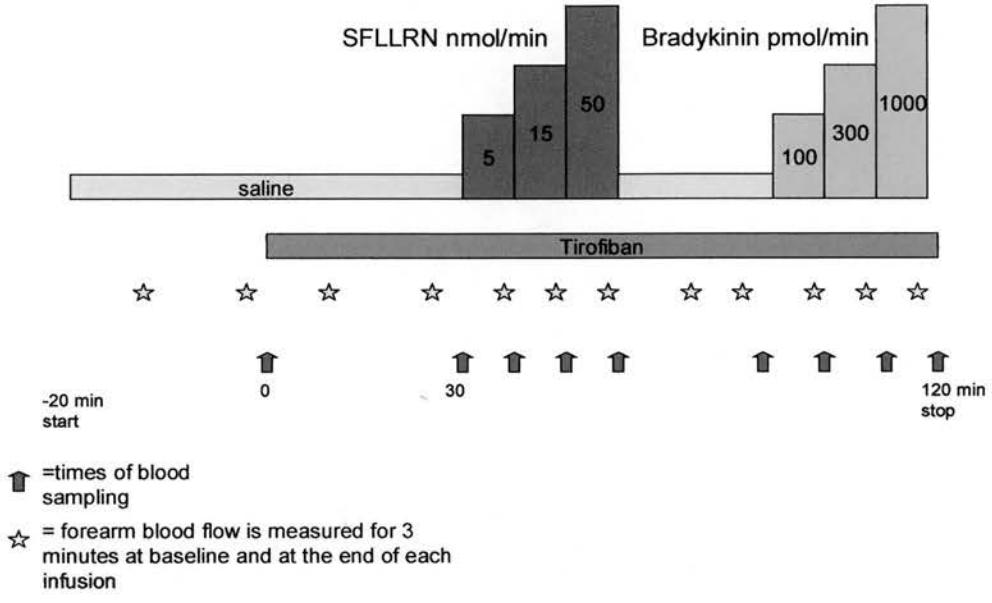

Figure 4.1 Schematic view of study *Protocol 3*.

Protocol 4: PAR-2 activation

Following a 20-minute baseline saline infusion, the PAR-2 activating peptide SLIGKV was infused at 160, 360 and 800 nmol/min for 8 minutes at each dose separated by 6-minute saline washout infusions, and followed by a 30 minute saline infusion before infusing bradykinin at 100, 300 and 1000 pmol/min [Witherow *et al*, 2002] for 8 minutes at each dose.

4.3.5 DATA ANALYSIS AND STATISTICS

Dorsal hand venous [Haynes *et al*, 1995] and forearm plethysmographic [Newby *et al*, 1997a] data were analysed as described previously. Variables are reported as means±SEM and analysed using repeated measures one- or two-way ANOVA with post-hoc Bonferroni corrections and two-tailed Students *t*-test as appropriate. Statistical analysis was performed with GraphPad Prism (Graph Pad Software) and statistical significance taken at $P<0.05$.

4.4 RESULTS

4.4.1 *IN VIVO* EFFECTS OF PAR AGONISM ON DORSAL HAND VEINS

After venoconstriction (50-70%) was induced and maintained with norepinephrine, SFLLRN and SLIKGV caused dose-dependent venoconstriction ($n=8$, $P<0.001$; one-way ANOVA) and venodilatation ($n=6$, $P<0.001$; one-way ANOVA) respectively. In the absence of norepinephrine, SFLLRN caused a dose-dependent venoconstriction ($P<0.001$; one-way ANOVA) that was able to induce complete constriction of the venous segment. This was not mediated by platelet aggregation since co-infusion of tirofiban had no effect on SFLLRN-induced venoconstriction (Figure 4.2). SFLLRN was well tolerated by all subjects with no adverse effects. Vein patency was maintained at all times with no clinically apparent *in situ* thrombus formation.

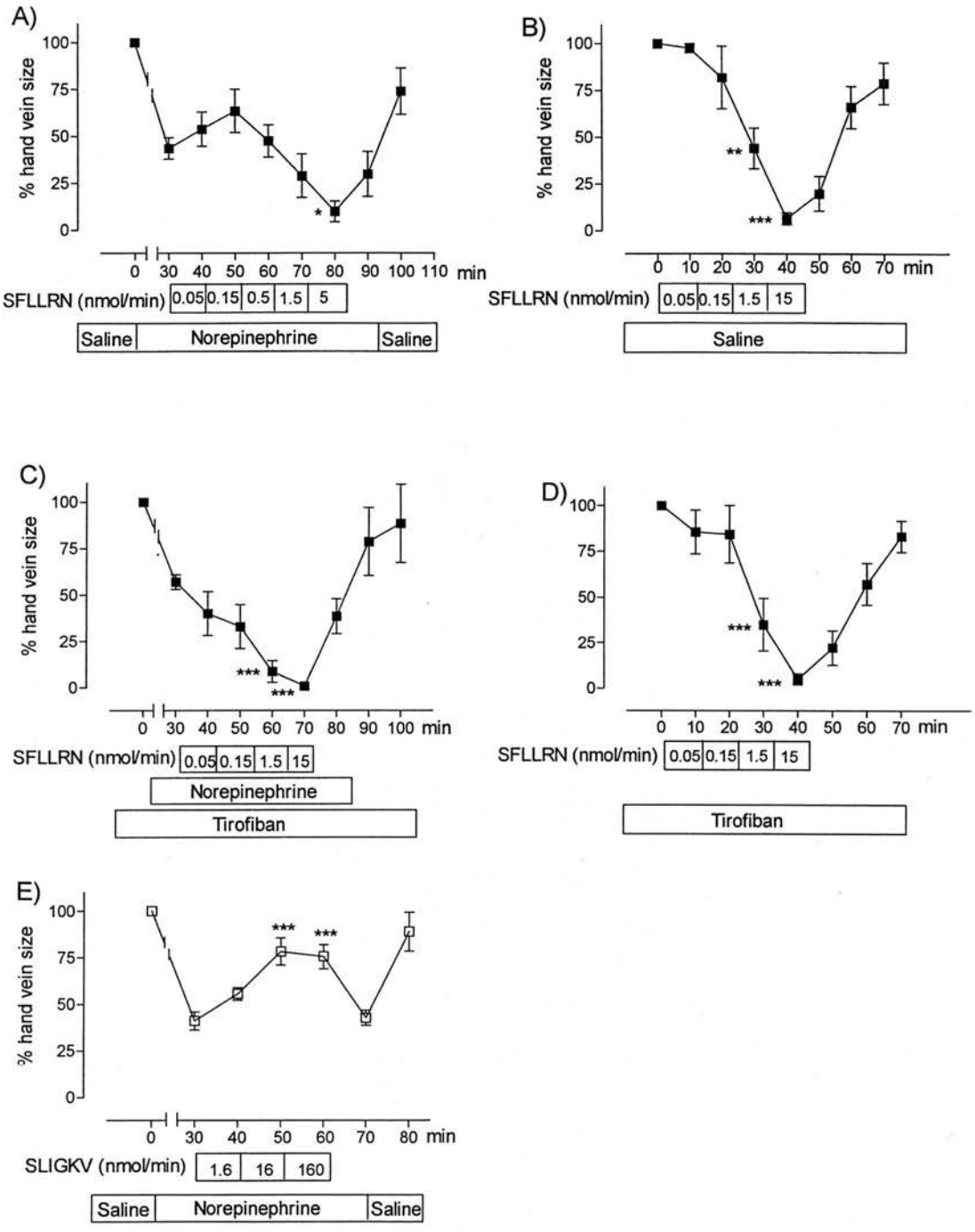

Figure 4.2 The PAR-1 activating peptide SFLLRN causes concentration-dependent venoconstriction (■, n=6-8, P<0.001; one-way ANOVA) that is unaffected by norepinephrine (A and B) or tirofiban (C and D) infusion, whereas the PAR-2 activating peptide SLIGKV (E) induces venodilatation (□, n=6, P<0.001; one-way ANOVA). Mean±SEM. *P<0.05, **P<0.01, ***P<0.001.
PAR - protease-activated receptor; ANOVA - analysis of variance; SEM - standard error of the mean.

4.4.2 *IN VIVO* EFFECTS OF PAR AGONISM ON FOREARM RESISTANCE VESSEL TONE

There was no change in heart rate, blood pressure or non-infused forearm blood flow throughout either study. Intra-arterial tirofiban had no effect on resting forearm blood flow (P=NS). Both PAR-1 activation with SFLLRN and PAR-2 activation with SLIGKV caused dose-dependent vasodilatation (Figure 4.3A and 4.3C; n=8, P<0.001 and P<0.01 respectively; one-way ANOVA) with a rapid onset and offset of action. As anticipated, bradykinin increased forearm blood flow (Figure 4.3B; P<0.001; one-way ANOVA) and was unaffected by tirofiban co-infusion (Figure 4.3D; P=NS *versus* no tirofiban).

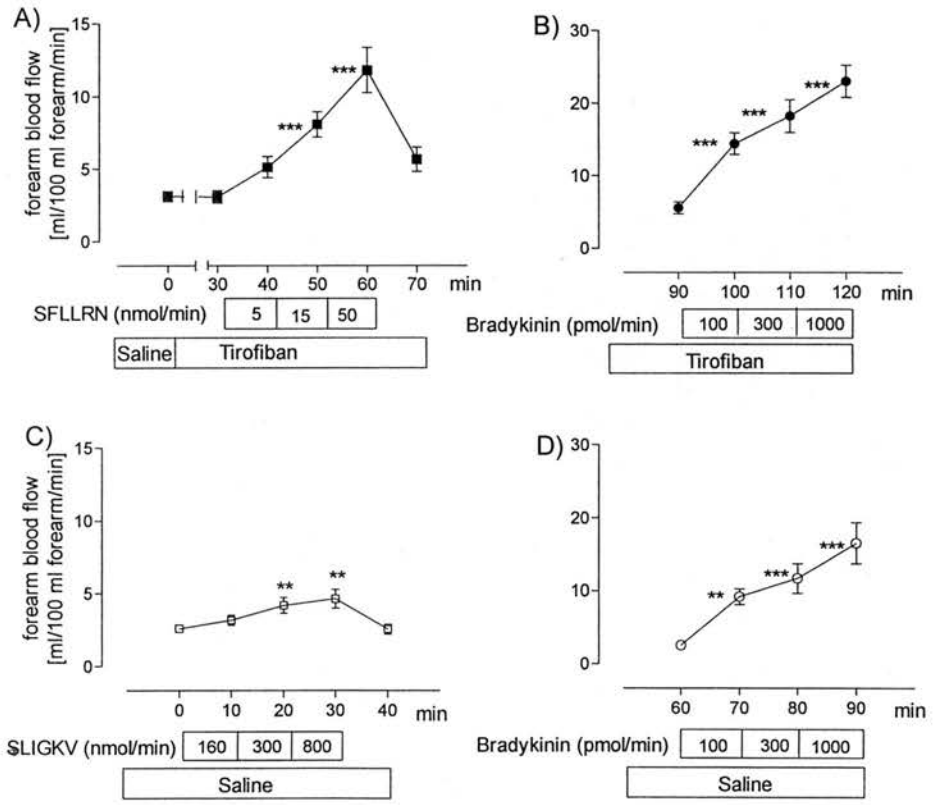

Figure 4.3 Forearm vasodilatation induced by the PAR-1 activating peptide SFLLRN (A; ■, n=8, P<0.001; one-way ANOVA), the PAR-2 activating peptide SLIGKV (C; □, n=8, P<0.01; one-way ANOVA), and bradykinin (B;● and D;○, n=8, P<0.001; one-way ANOVA). Mean±SEM. *P<0.05, **P<0.01, ***P<0.001.
PAR - protease-activated receptor; ANOVA - analysis of variance.

4.4.3 *IN VIVO* EFFECTS OF PAR AGONISM ON ENDOTHELIUM-DERIVED FACTORS

Plasma t-PA antigen and activity concentrations increased in a dose-dependent manner during SFLLRN and bradykinin, but not SLIKGV infusion (Figure 4.4; $P<0.001$ for all). Plasma PAI-1 antigen and activity, and vWF concentrations were unaffected by all infusions (P=NS for all; Table 4.2). Although there was an apparent rise in plasma PAI-1 antigen concentration at 50 nmol/min of SFLLRN, this did not achieve statistical significance (P=0.08, paired *t*-test *versus* baseline) and was not associated with increases in PAI-1 activity or vWF. There were no differences in peripheral blood haematocrit (0.416±0.006 *versus* 0.426±0.008) or platelet counts ($213\pm16 \times 10^9$ *versus* $216\pm15 \times 10^9$ /L) at baseline and the end of the study. The PAR-2 activating peptide, SLIKGV had no effect on plasma t-PA concentrations (P=NS for all; data not shown).

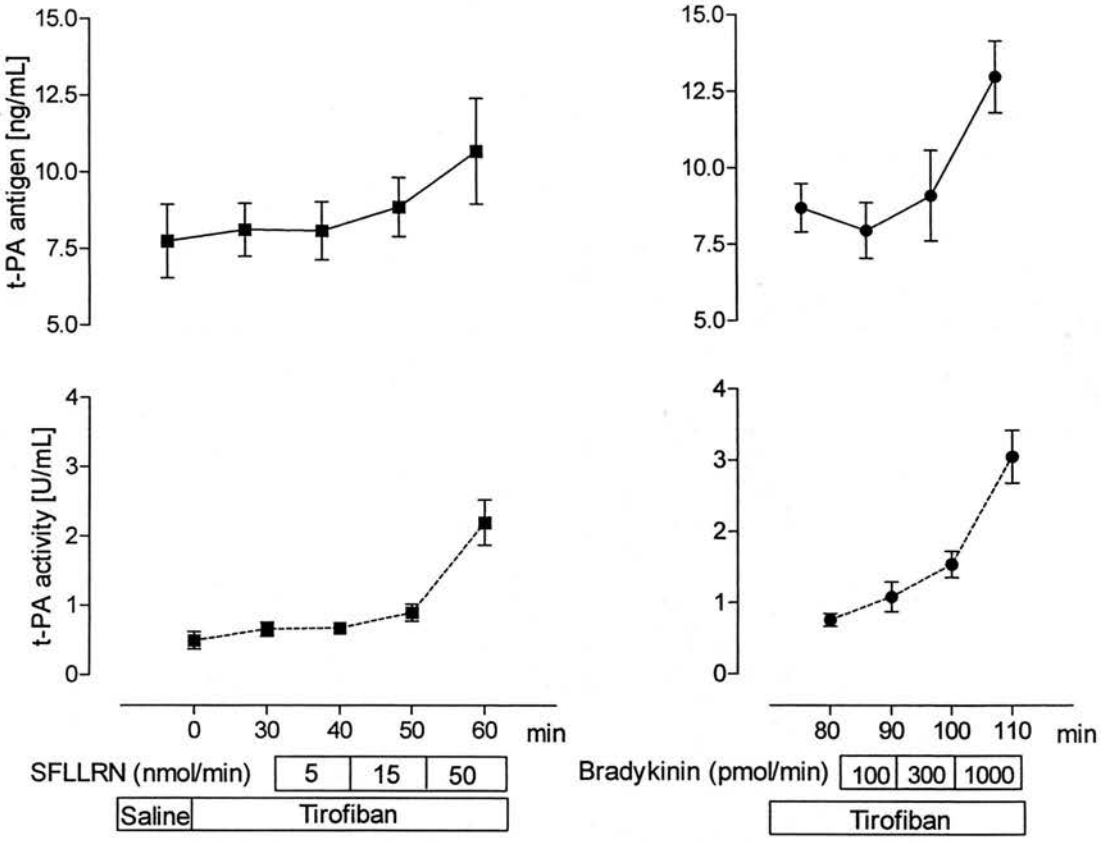

Figure 4.4 Plasma t-PA antigen (solid lines) and activity (dashed lines) during infusion of the PAR-1 activating peptide SFLLRN (■) and bradykinin (●). Mean±SEM.
t-PA - tissue plasminogen activator; PAR-1 - protease-activated receptor-1; SEM - standard error of the mean.

TABLE 4.2 Plasma PAI-1 (plasminogen activator inhibitor type 1) antigen and activity, and von Willebrand Factor (vWF) antigen concentrations in the infused and non-infused arms during brachial artery infusion of SFLLRN and bradykinin (n=8 for all). Mean±SEM (standard error of the mean)

	PAI-1 Antigen (ng/mL)		**PAI-1 Activity** (U/mL)		**vWF Antigen** (ng/mL)	
	Infused Arm	**Non-Infused Arm**	**Infused Arm**	**Non-Infused Arm**	**Infused Arm**	**Non-Infused** Arm
Baseline	27.3±5.5	27.7±5.6	0.58±0.14	0.51±0.13	0.72±0.08	0.72±0.07
Tirofiban	25.6±5.5	24.5±4.4	0.40±0.09	0.43±0.12	0.69±0.04	0.62±0.08
SFLLRN						
5 nmol/min	23.0±4.7	24.9±4.4	0.44±0.12	0.47±0.11	0.62±0.06	0.67±0.08
15 nmol/min	24.3±4.5	23.8±4.4	0.41±0.10	0.41±0.11	0.67±0.06	0.74±0.10
50 nmol/min	33.8±6.8	26.9±5.3	0.37±0.09	0.42±0.09	0.73±0.08	0.76±0.08
Washout	22.9±4.2	23.1±3.6	0.32±0.08	0.33±0.08	0.67±0.09	0.74±0.09
Bradykinin						
100 pmol/min	19.6±3.7	21.3±3.9	0.27±0.07	0.35±0.09	0.80±0.08	0.64±0.09
300 pmol/min	19.5±4.4	19.5±4.9	0.25±0.06	0.26±0.07	0.73±0.08	0.70±0.07
1000 pmol/min	19.5±3.2	19.1±2.9	0.16±0.03	0.25±0.05	0.76±0.07	0.67±0.07

4.4.4 *IN VIVO* EFFECTS OF PAR AGONISM ON PLATELET ACTIVATION

In keeping with results of previous *in vitro* studies, SFLLRN increased *in vivo* platelet-monocyte binding (from 16±5% to peak of 74±5%; Figure 4.5; P<0.0001; one-way ANOVA) that declined towards baseline with time.

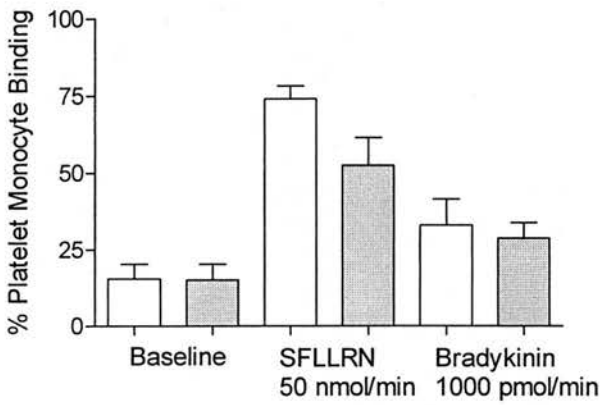

Figure 4.5 Platelet-monocyte binding during the infusion of the PAR-1 agonist SFLLRN in the infused (open bars) and non-infused (grey bars) arms. Mean±SEM.
PAR-1 - protease-activated receptor-1; SEM - standard error of the mean.

4.5 DISCUSSION

In a series of clinical studies we have, for the first time, described the direct vascular effects of PAR-1 agonism and established the induction of *in vivo* platelet activation, venoconstriction, vasodilatation and t-PA release as well as confirming the vasodilatory effects of PAR-2 agonism. These unique and contrasting effects of PAR-1 activation provide important insights into the physiological and pathophysiological role of thrombin in the venous and arterial circulations of man.

4.5.1 VASCULAR EFFECTS OF PAR ACTIVATING PEPTIDES

Protease-activated receptor-1 appears to be the principal receptor responsible for the vascular actions of thrombin and includes the regulation of vasomotion, platelet aggregation, inflammation and angiogenesis. Investigation of the *in vivo* vascular effects of thrombin itself presents challenges because of the direct activation of the coagulation cascade and platelets that may result in intravascular thrombosis. We have here, chosen to use PAR activating peptides that cause direct cellular stimulation without enzymatic activation of the coagulation cascade.

The direct vascular effects of PAR-1 receptor agonism have not been previously reported in man. Our study protocol was vigilant to minimise any potential prothrombotic effects associated with PAR-1 induced platelet aggregation. We defined the *in vitro* concentrations of GPIIb/IIIa receptor antagonism that would permit the direct *in vivo* intravascular infusion of PAR-1 activating peptide without inducing platelet aggregation and potential thrombosis. This appears to have been successfully achieved given the absence of significant adverse clinical side-effects or thrombosis.

Venous effects of PAR activating peptides

Thrombin, and the PAR-1 agonist SFLLRN, have previously been shown to cause endothelium- and NO-dependent venous and arterial dilatation in canine and porcine ring segments [Ku and Zaleski, 1993; Tesfamariam *et al*, 1993]. Although confirming previous observations of PAR-2 mediated venodilatation [Robin *et al*, 2003], we have rather unexpectedly described a dose-dependent venoconstriction

with PAR-1 activation. This may be mediated by platelet activation, release of endothelium-derived vasoconstrictors or a direct effect on venous smooth muscle cells. Neither norepinephrine nor tirofiban co-infusion appeared to affect this potent venoconstrictor effect. In combination with the apparent absence of *in situ* thrombosis, this suggests that PAR-1 induced venoconstriction was not mediated by platelet aggregation although we cannot preclude an effect of platelet activation.

Arterial effects of PAR activating peptides

In contrast to the venous effects, both PAR-1 and PAR-2 activation caused dose-dependent vasodilatation of forearm resistance vessels. We have not assessed the mechanism of this vasodilatation but vasomotor studies, and animal and clinical models suggest that this is likely to be endothelium- and NO-dependent [Yang *et al* 1994; Cheung *et al*, 1998; Robin *et al*, 2003]. However, data from animal studies have limited relevance in man because of the wide species variability in PAR-1 receptor expression and function. The mechanism of PAR-1 induced vasodilatation needs to be addressed in future clinical studies.

Protease-activated receptor expression varies between endothelial cell cultures originating from different human blood vessels [Fujiwara *et al*, 2004] and this may partly explain the contrasting responses between PAR-1 and PAR-2 in the arterial and venous circulations. Our findings that PAR-2 activation causes venous and arterial dilatation are consistent with previous findings [Robin *et al*, 2003], and supports the vascular role of PAR-2 receptors in inflammatory conditions.

4.5.2 Effects of PAR activating peptides on endothelium-derived factors

Intra-arterial PAR-1, but not PAR-2, agonism caused an acute dose-dependent increase in local endothelial t-PA release. In contrast to *in vitro* human endothelial culture studies [Cleator *et al*, 2005], this occurred in the absence of the release of other endothelium-derived factors, such as PAI-1 and vWF. However, there was an apparent rise in plasma PAI-1 antigen (P=0.08) with high dose PAR-1 agonism, but given the absence of an effect on PAI-1 activity and vWF, this may reflect the associated platelet activation and release of platelet-derived PAI-1. Plasminogen activator inhibitor type 1 is stored in platelet α-granules where its activity is ~5% of plasma due to the absence of the stabilising effect of vitronectin. Thus, PAR-1 agonism appears to have a selective pro-fibrinolytic effect on the arterial endothelium.

4.5.3 Effects of PAR activating peptides on platelet activation

Inhibition of platelet activation has been of major benefit in the prevention and treatment of cardiovascular diseases. Thrombin is one of the most potent physiological stimulants of platelet activation that appears to be mediated largely through the PAR-1 receptor although major contributions are also provided by PAR-4 and GPIb receptors. To date, strategies to reduce the action of thrombin have focused on direct inhibition of its enzymatic activity. Recently, PAR-1 antagonists have been developed as potential antiplatelet agents [Chackalamannil *et al*, 2005] that do not affect the coagulation cascade. It is therefore important to define the

in vitro and *in vivo* effects of PAR-1 agonism in man. As anticipated, we have demonstrated that PAR-1, but not PAR-2, agonism caused concentration-dependent platelet activation and aggregation. Moreover, in the presence of complete inhibition of platelet aggregation with the GPIIb/IIIa receptor antagonist tirofiban, PAR-1 agonism continued to induce platelet activation as measured by platelet-monocyte binding both *in vitro* and *in vivo*. This is consistent with our previous findings that whilst GPIIb/IIIa receptor antagonism inhibits platelet aggregation, it does not affect platelet activation or platelet-monocyte binding [Sarma *et al*, 2002]. The stimulatory effects of PAR-1 activation have previously been studies *in vitro* but here we demonstrate that PAR-1 activation *in vivo* causes marked platelet activation as measured by levels of platelet-monocyte binding. To enable us to carry out this study, tirofiban was infused throughout to inhibit the formation of platelet aggregates and prevent intra-arterial thrombosis, and doses based on previous *in vitro* studies.

4.5.4 PHYSIOLOGICAL SIGNIFICANCE OF VASCULAR PAR-1 ACTIVATION

We have, for the first time, described the unexpected and contrasting vascular effects of PAR-1 agonism *in vivo* in man. How do we interpret these effects? In an intact normal vessel, homeostatic mechanisms attempt to maintain vessel patency and minimise intravascular thrombus formation. Dorsal hand veins do not have resting tone and the induction of venodilatation will not affect venous blood flow. Therefore in the presence of developing venous thrombosis, venodilatation would not be beneficial whereas venoconstriction will potentially limit thrombus propagation and embolisation. In contrast, in the arterial circulation, it would be anticipated that increasing blood flow and endogenous fibrinolysis would limit arterial thrombosis by

ensuring rapid clearance and dissolution of a developing thrombus. We therefore propose that the vascular effects of PAR-1 agonism *in vivo* in man can be understood in terms of limiting intravascular thrombosis and maintaining vessel patency. We speculate that these physiological effects may be disturbed in patients with cardiovascular disease or prothrombotic disorders.

We conclude that PAR-1 agonism causes platelet activation, venoconstriction, vasodilatation and t-PA release *in vivo* in man. This has important implications in understanding the physiological vascular effects of thrombin as well as the pathogenesis of thromboembolic and atherothrombotic disorders.

CHAPTER 5

ROLE OF THE ENDOTHELIUM IN THE VASCULAR EFFECTS OF THE PROTEASE-ACTIVATED RECEPTOR-1 IN HUMANS

Guðmundsdóttir IL, Lang NN, Boon NA,
Ludlam CA, Webb DJ, Fox KA, Newby DE.
Role of the endothelium in the vascular effects of the thrombin receptor (protease-activated receptor type 1) in humans.
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5.1 SUMMARY

Thrombin is central to the pathophysiology of atherothrombosis. Its cellular actions are mediated via PAR-1. Protease-activated receptor-1 activation causes arterial vasodilatation, venoconstriction, platelet activation and t-PA release in man. The aim of this study was to determine the role of the endothelium in the vascular actions of PAR-1 activation *in vivo* in man. Dorsal hand vein diameter was measured during infusion of the PAR-1 activating peptide SFLLRN in six healthy volunteers before and after endothelial denudation. Forearm arterial blood flow, plasma fibrinolytic factors and platelet activation were measured in twenty-four healthy volunteers during venous occlusion plethysmography. The effects of inhibition of prostacyclin, NO and EDHF on PAR-1 responses were assessed during co-administration of aspirin, the 'nitric oxide clamp' L-NMMA and sodium nitroprusside, and TEA respectively. Endothelial denudation did not affect PAR-1 evoked venoconstriction (SFLLRN; 0.05-15 nmol/min). Although aspirin had no effect, SFLLRN-induced vasodilatation (5-50 nmol/min) was attenuated by the 'nitric oxide clamp' ($P<0.0001$) and TEA ($P<0.05$), and abolished by their combination ($P<0.01$). The 'nitric oxide clamp' augmented SFLLRN-induced t-PA and PAI-1 antigen ($P<0.0001$) release whilst TEA and aspirin had no effect. SFLLRN-induced platelet activation was unaffected by NO or prostacyclin inhibition. We conclude that acting via PAR-1, thrombin causes contrasting effects in the human vasculature and has a major interaction with the endothelium. This highlights the critical importance of

endothelial function during acute arterial injury and intravascular thrombosis, as occurs in cardiovascular events including myocardial infarction and stroke.

5.2 INTRODUCTION

Thrombin plays a central role in the coagulation cascade and thrombosis [Patterson *et al*, 2001]. It is one of the most powerful physiological agonists in the cardiovascular system and its actions are fundamental to the processes of atherosclerosis and its thrombotic consequences.

In addition to the enzymatic generation of fibrin, thrombin stimulates a range of cell types including platelets, endothelium and vascular smooth muscle cells. An extensive search for thrombin receptors ultimately culminated in the identification of a group of G-protein coupled receptors termed protease-activated receptors. These receptors are characterised by a unique mechanism of activation whereby the receptor undergoes proteolytic cleavage, unmasking a short peptide sequence that remains tethered and auto-activates the receptor [Vu *et al*, 1991; Swift *et al*, 2006]. To date, four different types of PARs have been identified: PAR-1, -3 and -4 are all activated by thrombin. PAR-2 is mainly activated by trypsin but transactivation of PAR-2 by cleaved PAR-1 has been recognised [O'Brien *et al*, 2000; Hollenberg and Compton, 2002].

Protease-activated receptors-1 are the principal thrombin receptors in man and have been extensively studied in small animals and cell cultures suggesting a diverse and

important role in various organs. Their activation stimulates a network of G-protein coupled signalling pathways that involve phospholipase Cβ, protein kinase C, calcium release, MAP kinases and potassium channels [Osssovskaya and Bunnett, 2004; Coughlin, 2006]. However, there is significant species heterogeneity with pre-clinical studies of limited relevance to man [Kinlough-Rathbone *et al*, 1993]. Exploring the role of PAR-1 receptors in the human vasculature, and the mechanisms involved, would deepen our understanding of the physiological role of thrombin and be important in the clinical development of new therapeutic strategies.

To understand the physiological actions of thrombin in the human vasculature is challenging because direct thrombin instillation has the potential to cause acute thrombosis *in situ* and hence vascular occlusion. The use of a PAR-1 activating peptide, however, permits the direct assessment of cellular responses to thrombin without the enzymatic activation of the coagulation cascade and fibrin formation. Using the short peptide mimetic SFLLRN, we have recently described the *in vivo* effects of PAR-1 activation in platelets, endothelium and vascular smooth muscle in man. For the first time, we were able to show that PAR-1 activation has unique and contrasting effects in the human vasculature including arterial dilatation, venous constriction, platelet activation and t-PA release [Guðmundsdóttir *et al*, 2006]. Given the central role of thrombin in the pathophysiology of cardiovascular disease, it is important to establish the mechanisms of these PAR-1 mediated effects and, in particular, the role of the endothelium. We therefore set out to explore the role of the endothelium in the vascular actions of PAR-1 activation *in vivo* in man.

5.3 METHODS

5.3.1 SUBJECTS

Thirty healthy non-smokers (mean age 22 years; range 19-37 years) were recruited into the study. The study was approved by the Lothian Research Ethics Committee and conducted in accordance with the Declaration of Helsinki and with the written informed consent of all volunteers. Participants were screened and excluded for clinically significant conditions including hypertension, hyperlipidaemia, diabetes mellitus, asthma and coagulopathy. No participant had suffered a recent infective or inflammatory condition or had taken any medications in the 7 days prior to the study.

All studies were carried out in a quiet, temperature controlled room (22-24°C). Participants were semi-recumbent (venous studies) or supine (arterial studies) and had abstained from alcohol for 24 hours, and from food and caffeine-containing drinks for at least 4 hours prior to the study.

5.3.2 DRUGS

Custom synthesised clinical grade SFLLRN, bradykinin and L-NMMA and TEA were purchased from Clinalfa, Laufelfingen, Switzerland. Acetylcholine was from Novartis Pharmaceuticals UK Ltd, Frimley, UK, sodium nitroprusside from David Bull Laboratories, Faulding, UK, norepinephrine from Abbott Laboratories, Maidenhead, UK, and tirofiban from Merck, Sharp and Dohme, Hoddesdon, UK.

Infused drugs were all administered following dissolution in 0.9% saline. All solutions were freshly prepared on the day of the study.

5.3.3 VENOUS STUDIES

A 23-gauge needle was sited in a dorsal hand vein and total infusion rate kept constant at 0.25 mL/min in all studies. The hand was supported above the level of the heart and an upper arm cuff inflated to 40 mmHg to obstruct venous return. The internal diameter of the dorsal hand vein was measured by the Aellig technique [Aellig, 1981] in six healthy volunteers. In brief, a magnetised lightweight rod rested on the summit of the infused vein approximately 1 cm downstream from the tip of the infusion needle. The rod passes through the core of a LVDT, supported above the hand by a small tripod. Changes in diameter of the vein cause vertical displacement of the rod, leading to a linear change in the voltage generated by the transformer. This enables calculation of absolute changes in vein size.

Protocol 1: Venous effects of PAR-1 activation

First we established the presence of functional endothelium. As dorsal hand veins do not have resting tone, norepinephrine (1-128 ng/min) was used to induce 70% reduction in vein diameter. Once stable venoconstriction was obtained, acetylcholine (1 nmol/min; Novartis Pharmaceuticals UK Ltd, Frimley, UK) was co-infused with norepinephrine for 8 minutes to demonstrate endothelium-dependent venodilatation and an intact, functional endothelium. Following a 20-minute saline infusion, the PAR-1 activating peptide, SFLLRN (0.05-15 nmol/min; Clinalfa, Switzerland), was administered intravenously before a final 20-minute saline washout infusion.

Protocol 2: Venous effects of PAR-1 activation following endothelial denudation

At the end of Protocol 1, the endothelium of the venous segment was denuded as previously described [Sogo *et al*, 2000b]. In brief, a second 23-gauge butterfly needle was sited in the opposite direction, 3-4 cm downstream from the tip of the infusion needle, and this segment of the vein isolated by the use of occlusion wedges. Distilled water was infused through the venous segment at a rate of 5 mL/min for 15 minutes, thereby causing endothelial denudation that persists for at least 2 days [Sogo *et al*, 2000b]. Aspirin (300 mg orally), was given 30 minutes prior to start of the first study, and on each of the two subsequent days to prevent venous thrombosis. On the second day following denudation, subjects re-attended and *Protocol 1* was repeated.

5.3.4 ARTERIAL STUDIES

All subjects underwent cannulation of the brachial artery with a 27-gauge standard wire steel needle under controlled conditions. The intra-arterial infusion rate was kept constant at 1 mL/min throughout all studies. Forearm blood flow was measured in the infused and non-infused arms by venous occlusion plethysmography using mercury-in-silastic strain gauges as described previously [Newby *et al*, 1997a; Newby *et al*, 1999]. Supine heart rate and blood pressure were monitored at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer. Tirofiban (1.25 μg/min) was co-infused during the studies to inhibit potential PAR-1 activation induced platelet aggregation *in vivo* [Guðmundsdóttir *et al*, 2006]. This dose of tirofiban does not affect

platelet-monocyte binding, forearm blood flow or baseline concentration of t-PA [Guðmundsdóttir *et al*, 2006].

Protocol 3: Role of nitric oxide and prostacyclin in PAR-1 induced vasodilatation

Forearm blood flow was measured by venous occlusion plethysmography in response to brachial artery infusion of SFLLRN (PAR-1 agonist; 5-50 nmol/min) with tirofiban (1.25 μg/min) in eight healthy volunteers on four visits using a randomised controlled cross-over study employing a two-by-two factorial design: with and without aspirin (600 mg orally; to inhibit prostacyclin synthesis) and the 'nitric oxide clamp'. Assuming total forearm blood flow of 25 mL/min, this will achieve end-organ concentrations of 0.2-2.0 μM SFLLRN.

The 'nitric oxide clamp' was used to determine the contribution of NO in PAR-1 mediated vascular effects. Following baseline intra-arterial tirofiban infusion, the NO synthase inhibitor, L-NMMA (8 μmol/min), was co-infused. To compensate for L-NMMA induced basal vasoconstriction, forearm blood flow was returned to baseline using a titrated dose of exogenous NO in the form of intra-brachial sodium nitroprusside (90-900 ng/min). This dose of sodium nitroprusside was co-infused with L-NMMA and continued throughout the study. This arrangement allows a constant 'clamped' delivery of exogenous NO whilst endogenous NO synthase activity is inhibited.

Protocol 4: Role of calcium-activated potassium channels/EDHF in PAR-1 induced vasodilatation

Forearm blood flow was measured in a further eight healthy volunteers in whom intrabrachial SFLLRN (5-50 nmol/min), bradykinin (30-300 pmol/min) and sodium nitroprusside (2-8 μg/min) were co-infused with either saline placebo or TEA (1 mg/min) on either of two visits using a randomised double blind cross-over design. Again, agonists were co-infused with intra-arterial tirofiban (1.25 μg/min), which was continued throughout the study. At the dose used, TEA is a non-selective potassium channel antagonist [Champion *et al*, 1997; Honing *et al*, 2000; Inokuchi *et al*, 2004].

Protocol 5: Role of endothelium-dependent vasodilators in PAR-1 induced vasodilatation

In the final series of studies, TEA or saline placebo was co-infused with ascending doses of bradykinin and SFLLRN in eight volunteers using a randomised double blind cross-over design. In this series, EDHF activity was isolated by inhibiting NO and prostacyclin production on both visits. The 'nitric oxide clamp' was employed as described above (*Protocol 3*), and cyclooxygenase activity was inhibited with a single 600 mg dose of oral aspirin 1 hour prior to each study.

Blood sampling

Seventeen-gauge venous cannulae were inserted into left and right antecubital fossae. Blood samples were drawn simultaneously from each arm at baseline. Blood samples were also drawn before SFLLRN or bradykinin infusion and after each dose

of SFLLRN or bradykinin. Blood was collected into acidified buffered citrate (Stabilyte, Biopool International, UK; for t-PA assays) and into citrate (Becton Dickinson Vacutainer, BD UK Ltd, UK; for PAI-1, β-thromboglobulin and vWF assays). Samples were kept on ice before centrifugation at 2000 *g* for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma t-PA antigen and activity (t-PA Combi Actibind Elisa Kit; Technoclone, Austria), PAI-1 antigen and activity (Elitest-PAI-1 Antigen and Zymutest PAI-1 Activity; Hyphen Biomed, France), β-thromboglobulin (Asserachrom Btg, Diagnostica Stago, France) and vWF (Dako, Glostrup, Denmark) concentrations were determined by enzyme-linked immunosorbant assays. Full blood count was measured at baseline and at the end of the study.

Platelet-monocyte binding

In *Protocols 3* and *4*, blood was collected from each arm for determination of platelet-monocyte binding at baseline and after the highest dose of SFLLRN. Five millilitres of venous blood were collected and transferred into a tube containing the direct thrombin inhibitor, PPACK. Five minutes after sampling, blood was incubated with appropriate monoclonal antibodies labelled with fluorochromes for 20 minutes and platelet-monocyte aggregates measured as described previously [Harding *et al*, 2004].

5.3.5 DATA ANALYSIS AND STATISTICS

Dorsal hand venous [Haynes *et al*, 1995] and forearm plethysmographic [Newby *et al*, 1997a] data were analysed as described previously. Variables are reported as

means±SEM and analysed using repeated measures ANOVA with post-hoc Bonferroni corrections and two-tailed Students *t*-test as appropriate. Statistical analysis was performed with GraphPad Prism (Graph Pad Software) and statistical significance taken at the 5% level.

5.4 RESULTS

5.4.1 ENDOTHELIUM AND PAR-1 INDUCED VENOCONSTRICTION

The role of the endothelium in PAR-1 induced vasomotor effects was assessed by comparing venous responses before and after local endothelial denudation. This was achieved through brief instillation of distilled water in an isolated dorsal hand vein segment. The dorsal hand vein was pre-constricted with norepinephrine to achieve approximately 70% constriction (35±4% before denudation, 33±7% on the second visit), the dose of norepinephrine required to achieve this constriction appeared slightly higher on the second visit although not statistically significant (6±2 versus 13±6 ng/min, P=0.2), which is in keeping with previous publications [Sogo et al, 2000b]. After pre-constriction with norepinephrine, the presence or absence of functional endothelium was confirmed by the co-infusion of acetylcholine (1 nmol/min). Acetylcholine caused venodilatation in the presence of endothelium and venoconstriction in its absence (Figure 5.1; from 35±4% to 55±7% in the presence of endothelium *versus* 33±7% to 18±6% in the absence of endothelium; P<0.01 for both; ANOVA). After endothelial denudation, there appeared to be a trend toward enhanced venoconstriction induced by the PAR-1 activating peptide, SFLLRN (Figure 5.1; P=0.09; ANOVA).

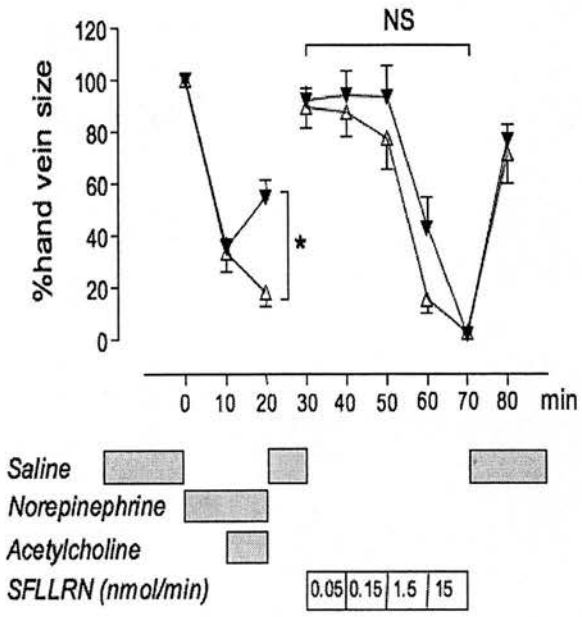

Figure 5.1 Dorsal hand vein responses to acetylcholine and SFLLRN (after pre-constriction with norepinephrine). Before (solid triangles) and after (open triangles) endothelial denudation. *P<0.05 and NS=non-significant (P=0.09); presence *versus* absence of the endothelium (ANOVA).
ANOVA - analysis of variance.

5.4.2 ENDOTHELIUM-DERIVED VASODILATORS AND PAR-1 INDUCED VASODILATATION

SFLLRN caused an increase in forearm blood flow that was unaffected by prostacyclin inhibition with oral aspirin (600 mg; Figure 5.2A). The NO synthase inhibitor, L-NMMA, caused ~50% decrease in basal forearm blood flow (from 3.04±0.37 to 1.49±0.19 mL/100mL tissue/min; $P<0.001$). Intra-brachial sodium nitroprusside (90-900 ng/min), an exogenous NO donor, was titrated to restore forearm blood flow back to baseline levels (2.77±0.24 mL/100mL tissue/min; $P=0.46$ clamp dose sodium nitroprusside *versus* baseline; paired Student's *t*-test). The inhibition of endogenous NO synthesis by the 'nitric oxide clamp' attenuated SFLLRN-induced vasodilatation (Figure 5.2B).

Potassium channel antagonism with TEA (1 mg/min) did not affect baseline blood flow ($P=0.76$; data not shown). It attenuated vasodilatation to SFLLRN (Figure 5.2C) whilst the combination of prostacyclin, NO synthase and potassium channel inhibition appeared to abolish SFLLRN-induced vasodilatation (Figure 5.2D).

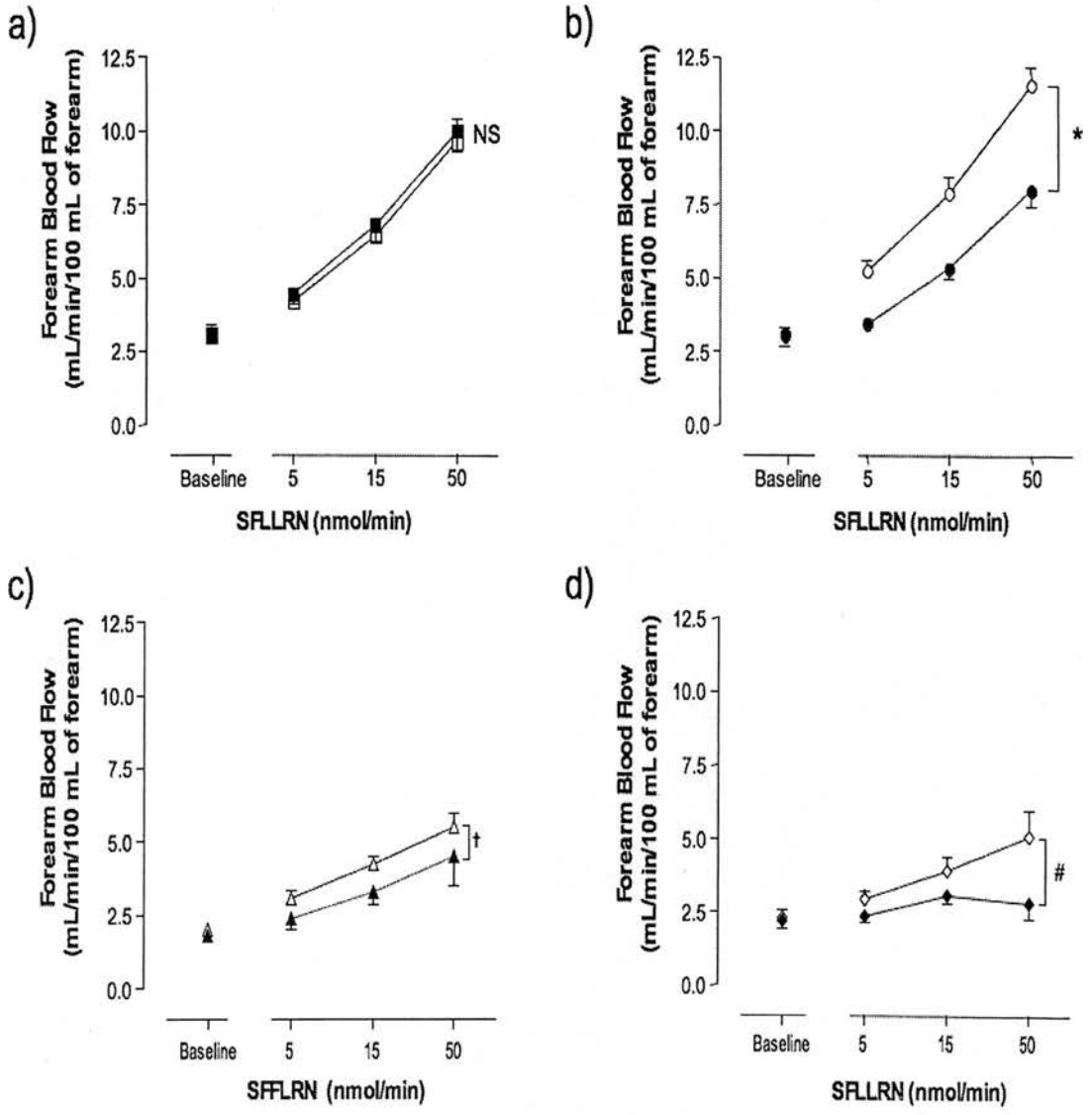

Figure 5.2 SFLLRN-induced forearm arterial vasodilatation. In the presence (closed symbols) and absence (open symbols) of A) aspirin (squares); NS=non-significant (P=0.53) in the presence *versus* the absence of aspirin; analysis of variance (ANOVA), B) the 'nitric oxide (NO) clamp' (circles); *P<0.0001 in the presence *versus* the absence of the 'NO clamp' (ANOVA), C) tetraethylammonium (TEA) (triangles); †P<0.05 in the presence *versus* the absence of TEA (ANOVA), and D) aspirin, the 'NO clamp' and TEA (diamonds).
#P<0.01 in the presence *versus* the absence of aspirin, the 'NO clamp' and TEA (ANOVA).

Both with and without concurrent NO synthase and prostacyclin synthase inhibition, TEA attenuated, but did not abolish arterial vasodilatation to the control endothelium-dependent vasodilator, bradykinin. (Figure 5.3A and 5.3B). Tetraethylammonium did not affect endothelium-independent forearm arterial vasodilatation to sodium nitroprusside (Figure 5.3C).

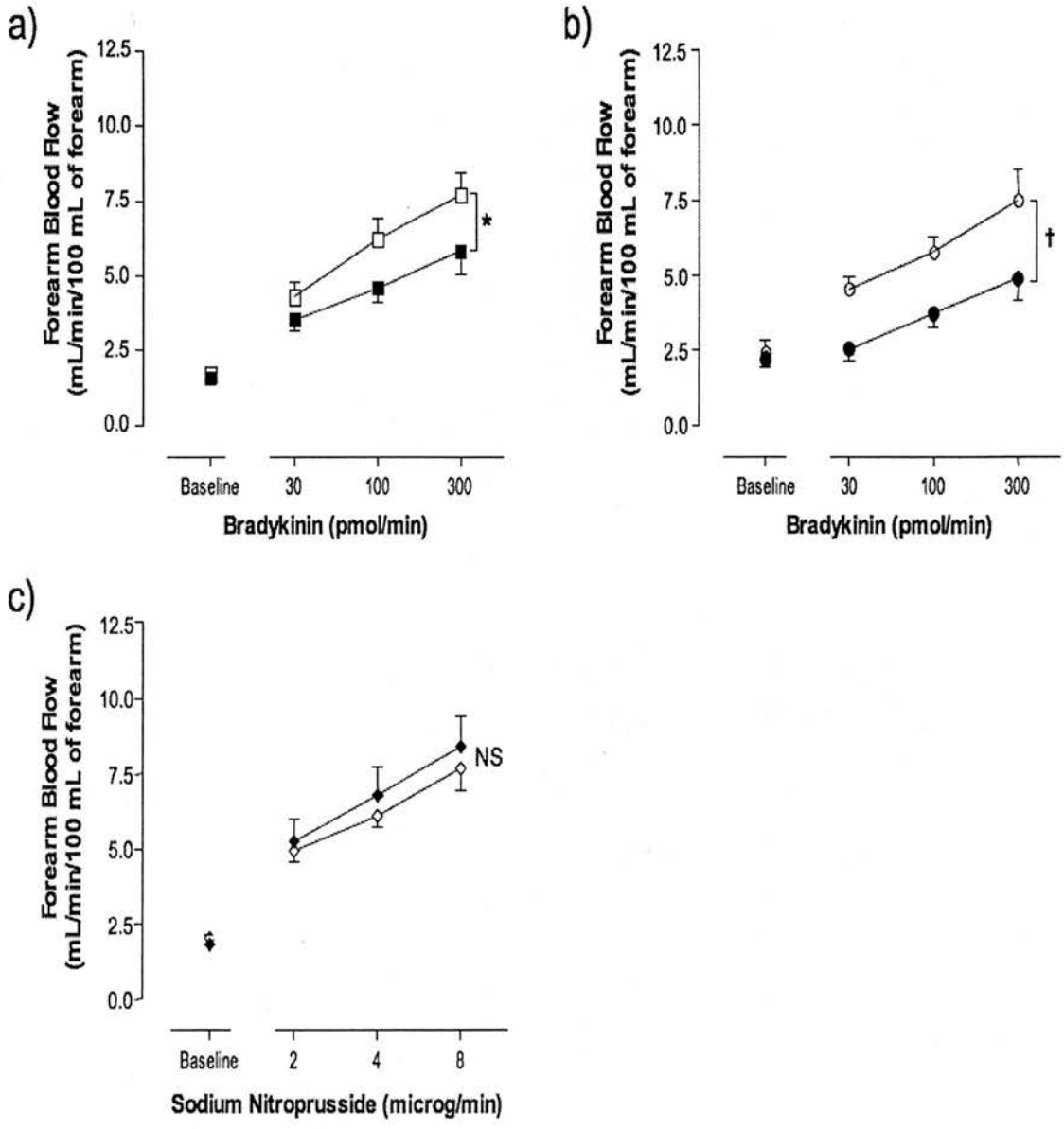

Figure 5.3 Bradykinin- and sodium nitroprusside-induced forearm arterial vasodilatation. Forearm arterial vasodilatation induced by (A) bradykinin (squares), (B) bradykinin in the presence of the 'nitric oxide clamp' and aspirin (circles) and (C) sodium nitroprusside (diamonds), in the presence (solid symbols) and absence (open symbols) of tetraethylammonium (TEA). *P<0.05, †P=0.0001, NS=non-significant (P=0.41) in the presence *versus* the absence of TEA (ANOVA).
ANOVA - analysis of variance.

5.4.3 ENDOTHELIUM-DERIVED VASODILATORS AND PAR-1 INDUCED RELEASE OF FIBRINOLYTIC AND COAGULANT FACTORS

SFLLRN increased net t-PA antigen and activity and PAI-1 antigen release but did not affect net PAI-1 activity (Figure 5.4). This increase was augmented by the 'nitric oxide clamp' (Figure 5.4) but not affected by aspirin or TEA (data not shown; P=NS; ANOVA). Bradykinin caused a dose-dependent increase in net t-PA antigen ($P<0.05$; ANOVA) and activity ($P<0.0001$; ANOVA) release but did not affect PAI-1 antigen and activity release (P=NS for both; ANOVA). Tetraethylammonium did not alter bradykinin-induced PAI-1 or t-PA release. Neither bradykinin nor SFLLRN affected vWF release (data not shown; P=NS; ANOVA).

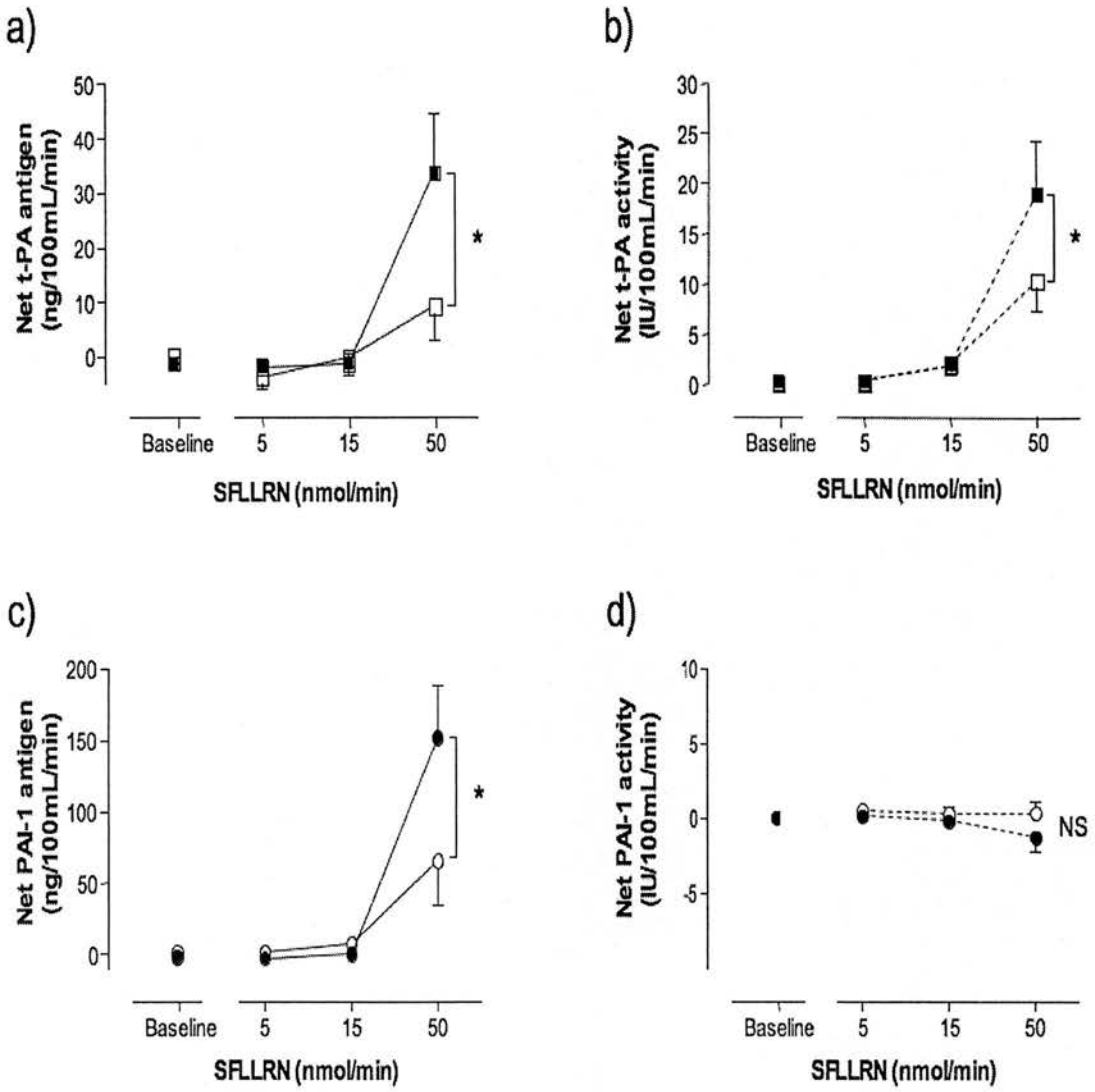

Figure 5.4 SFLLRN-induced tissue plasminogen activator and plasminogen activator inhibitor release. Net tissue plasminogen activator (t-PA; squares) and plasminogen activator inhibitor type 1 (PAI-1; circles) antigen (solid lines) and activity (dashed lines) in response to intra-brachial SFLLRN in the presence (solid symbols) and absence (open symbols) of the 'nitric oxide (NO) clamp'. *P<0.0001, NS=non-significant (P=0.075) in the presence *versus* the absence of the 'NO clamp' (ANOVA).
ANOVA - analysis of variance.

5.4.4 ENDOTHELIUM-DERIVED VASODILATORS AND PAR-1 INDUCED PLATELET ACTIVATION

SFLLRN increased platelet-monocyte binding but this was unaffected by inhibition of NO or prostacyclin (Table 5.1). In contrast, SFLLRN increased β-thromboglobulin (P<0.001; ANOVA) that was augmented during the 'nitric oxide clamp' (P<0.01; ANOVA; Figure 5.5) but unaffected by aspirin (P=NS; ANOVA).

TABLE 5.1 Per cent platelet-monocyte binding at baseline and after intrabrachial SFLLRN (50 nmol/min) in the infused and non-infused forearm in the presence and absence of aspirin and the 'nitric oxide clamp'. *P<0.001 *versus* baseline (ANOVA)

	PER CENT PLATELET-MONOCYTE BINDING			
	Baseline		**Post SFLLRN**	
	Infused Arm	***Non-Infused arm***	***Infused Arm***	***Non-Infused Arm***
Placebo	16.62±4.38	12.48±1.61	75.89±5.09*	41.36±6.86*
Aspirin only	14.43±1.95	14.87±3.61	72.07±5.41*	49.67±7.82*
NO clamp only	11.84±1.52	16.95±3.23	81.21±5.68*	46.42±7.42*
Aspirin + NO clamp	12.19±1.66	14.15±2.02	82.85±5.57*	46.63±6.48*

ANOVA - analysis of variance.

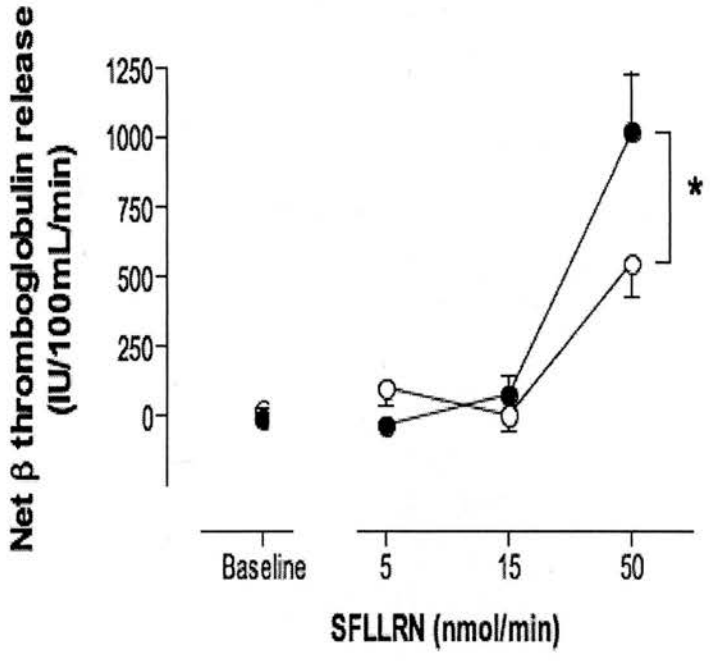

Figure 5.5 SFLLRN-induced ß-thromboglobulin release. In the presence (closed circles) and absence (open circles) of the 'nitric oxide (NO) clamp'. *P<0.01 net *ß*-thromboglobulin release induced by SFLLRN (50 nmol/min) in the presence *versus* the absence of the 'NO clamp', analysis of variance (ANOVA).

5.5 DISCUSSION

Thrombin is one of the most powerful physiological agonists in the cardiovascular system and its actions are fundamental to the processes of atherothrombosis. In a series of studies, we have described here the contrasting role of the endothelium in the PAR-1 mediated vascular actions of thrombin *in vivo* in man. Whilst not providing a major contribution to venoconstriction or PAI-1 release, the endothelium mediates PAR-1 induced arterial vasodilatation and t-PA release. Our findings provide clear evidence of a major interaction between the vascular endothelium and thrombin *in vivo* in man. Furthermore, it highlights the critical importance of endothelial function at the time of acute arterial injury and intravascular thrombosis, such as during acute coronary syndromes.

5.5.1 ROLE OF THE ENDOTHELIUM IN PAR-1 INDUCED VASOMOTION

We have previously described the unexpected finding of PAR-1 induced venoconstriction in man [Guðmundsdóttir *et al*, 2006]. Whilst not caused by platelet aggregation [Guðmundsdóttir *et al*, 2006], this effect could be mediated by either a direct action on vascular smooth muscle or via the release of endothelium-derived vasoconstrictors, such as endothelin or angiotensin II. To address this question, we assessed PAR-1 venoconstriction before and after endothelial denudation by instillation of distilled water. There was a modest trend towards enhanced venoconstriction after endothelial denudation and we cannot exclude a small contribution from the endothelium that may also include the release of venodilatory mediators such as nitric oxide. However, PAR-1 continued to induce a marked

dose-dependent venoconstriction even in the absence of the endothelium suggesting a dominant and direct effect of PAR-1 on the vascular smooth muscle cells.

In contrast to effects on the venous circulation, PAR-1 agonism causes potent arterial vasodilatation. This suggests a different effect on the arterial vasculature that is likely to be mediated by the endothelium. It would be difficult and ethically challenging to conduct comparable *in vivo* endothelial denudation studies in the arterial circulation of man. We chose, therefore, to use a pharmacological approach to the inhibition of the three main known mediators of endothelium-dependent vasodilatation: prostacyclin, NO and EDHF. Although prostacyclin inhibition appeared to have no effect, inhibition of NO and potassium channels both attenuated the PAR-1 induced vasodilatation. Consistent with some cross-talk and compensatory up-regulation, combined inhibition of all vasodilator mechanisms appeared to produce greater inhibition, if not abolition, of the vasodilator actions of PAR-1 agonism. This suggests that, unlike the venous circulation, PAR-1 mediated arterial actions are dominated by, and dependent upon, the vascular endothelium.

5.5.2 PAR-1 INDUCED RELEASE OF ENDOTHELIUM-DERIVED FACTORS

In addition to vasomotion, PAR-1 has important effects on the release of endothelium-derived coagulant and fibrinolytic factors. In keeping with a wide range of other endothelial G-protein coupled receptor dilator agonists [Oliver *et al*, 2005], we confirmed our earlier findings that PAR-1 agonism causes endothelial t-PA release without affecting vWF. However, we also report here that SFLLRN-induced t-PA release appeared to be augmented by the inhibition of endogenous NO

production. Smith *et al* [2003] have reported similar findings when they examined bradykinin evoked t-PA release in the presence and absence of L-NMMA. Because t-PA release is independent of NO and cyclooxygenase activity, it has been suggested that EDHF is responsible for its release [Brown *et al*, 2000]. One could speculate that, by inhibiting NO activity, EDHF is up-regulated and accounts for the augmented t-PA release induced by SFLLRN in our study and by bradykinin in Smith's study [Smith *et al*, 2003]. However, in contrast to TEAs inhibitory effects on SFLLRN-induced arterial vasodilatation, it had no effect on SFLLRN evoked t-PA release. Similar findings have recently been reported by Muldowney and colleagues [2007] who examined the role of EDHF in an *in vitro* model of thrombin-induced endothelial t-PA release in their study. A variety of potassium channel antagonists, including TEA, had no effect on thrombin-induced t-PA release whilst antagonists of specific EETs appeared to inhibit thrombin-induced release of t-PA.

One further novel finding in our study was the increase in PAI-1 release, especially during NO synthase inhibition. To date, there have been no reports of acute increases in plasma PAI-1 concentrations following the administration of endothelial agonists, especially using the forearm model [Oliver *et al* 2005]. Whilst the endothelium is an important source of PAI-1, we believe our findings are consistent with acute platelet release of PAI-1. There are several reasons to support our contention. First, although PAI-1 antigen concentrations increased, there was no corresponding rise in PAI-1 activity. Indeed, PAI-1 activity fell during marked release of t-PA. Plasminogen activator inhibitor type 1 is stored in platelet α-granules where its activity is very low (<5% of the activity seen in plasma) due to the absence of the stabilising effect of

vitronectin. In contrast, endothelial-derived PAI-1 would be anticipated to remain active. Second, there was no concurrent rise in vWF confirming a selective effect on the endothelium with isolated t-PA release. Third, we also demonstrated concomitant platelet activation with marked increases in platelet-monocyte binding and release of β thromboglobulin; the latter is also stored in the α-granules of platelet. Finally, PAR-1 induced PAI-1 release was augmented during the 'nitric oxide clamp'. Nitric oxide has important antiplatelet effects and, in the presence of its inhibition, increased platelet activation may have led to greater PAI-1 release.

5.5.3 Clinical relevance

Until recently, it has not been possible to undertake a safe clinical assessment of the vasomotor effects of thrombin due to its potent stimulatory effects upon the coagulation cascade. However, the synthetic activating peptide, SFLLRN, allows the examination of activation of the human PAR-1 thrombin receptor without activation of the coagulation cascade. This also permits the assessment of PAR-1 actions independent of the potential confounding effects that the activated coagulation pathway may have upon vascular responses.

We have demonstrated that many of the arterial effects of PAR-1 agonism are dependent upon, and mediated through, the endothelium and can therefore be used to assess endothelial function. To date, many endothelial G-protein coupled receptor agonists have been used to assess endothelial function, such as acetylcholine and substance P. However, such agents are unlikely to have a major role in vascular physiology or pathophysiology and, as pharmacological tools, their relevance to the

assessment of endothelial vasomotor function has limitations. As a more physiologically relevant tool, PAR-1 agonism may be a more appropriate method of assessing endothelial function in the context of atherothrombosis. These novel insights into the vascular actions of PAR-1 agonism will not only contribute to our understanding of human physiology and pathophysiology but also promises to inform the clinical development of novel antithrombotic PAR-1 receptor antagonists.

5.5.4 STUDY LIMITATIONS

We chose to use SFLLRN as a PAR-1 agonism for several reasons. First, the vast majority of published work has employed SFLLRN as a PAR-1 activating peptide and its actions have been widely characterised. Second, we have previous clinical experience of the *in vivo* actions of SFLLRN and this has facilitated the comparability of our current findings with our previous 'first-into-man' clinical studies. Finally, SFLLRN is identical to the active cleaved sequence of the human PAR-1 receptor and represents a more physiologically relevant agonist of the receptor.

Whilst SFLLRN is selective for the PAR-1 receptor, it does have agonist activity at the PAR-2 receptor: 4-fold greater selectivity for the PAR-1 *versus* PAR-2 receptor [Kawabata *et al*, 1999]. Therefore, there remains a possibility that a contribution of the observed actions of SFLLRN may represent PAR-2 agonism. However, we do not believe this is likely for several reasons. First, we have previously shown that SLIGKV, a highly selective PAR-2 activating peptide, causes only modest arterial vasodilatation at high doses and, in contrast to PAR-1 activation, causes marked

venodilatation and does not cause arterial t-PA release *in vivo* [Guðmundsdóttir *et al*, 2004]. Moreover, the predicted end-organ concentration of the highest dose of SFLLRN used in our current and previous studies is 4-fold lower than the EC_{50} for the PAR-2 receptor [Kawabata *et al*, 1999]. However, we do accept that, in future studies, consideration should be given to the use of the more selective PAR-1 activating peptide, TFLLRN [Kawabata *et al*, 1999; Osssovskaya and Bunnett, 2004].

5.5.5 Conclusions

Activation of PAR-1 causes contrasting effects in the human vasculature. It causes endothelium-dependent arterial vasodilatation and t-PA release as well as endothelium-independent venoconstriction and PAI-1 release. There appears to be a major interaction between the vascular endothelium and thrombin's PAR-1 mediated effects *in vivo* in man. This highlights the critical importance of endothelial function particularly at the time of acute arterial injury and intravascular thrombosis, such as occurs during many acute cardiovascular events including myocardial infarction and stroke.

CHAPTER 6

THE EFFECTS OF PROTEASE-ACTIVATED RECEPTOR-1 ACTIVATION ON VASCULAR RESPONSES AND FIBRINOLYSIS IN SMOKERS

6.1 SUMMARY

Cigarette smoking is associated with increased risk of atherothrombosis and impaired endogenous fibrinolysis. Activation of the thrombin receptor PAR-1 causes endothelium-dependent arterial vasodilatation and release of endogenous fibrinolytic factors. The aims of this study were to test the hypothesis that cigarette smoking adversely alters PAR-1 mediated vascular effects *in vivo* in man. Forearm blood flow was measured by venous occlusion plethysmography in twelve cigarette smokers and twelve age- and sex-matched non-smokers during intrabrachial infusions of PAR-1 activating peptide (SFLLRN; 5-50 nmol/min), bradykinin (100-1000 pmol/min) and sodium nitroprusside (2-8 μg/min). Plasma tissue plasminogen activator and PAI-1 antigen and activity concentrations were measured at regular intervals throughout. All agonists caused dose-dependent increases in forearm blood flow ($P<0.0001$ for all). Whilst bradykinin and sodium nitroprusside caused similar vasodilatation, SFLLRN-induced vasodilatation was attenuated in smokers ($P=0.04$). Smokers had modest reductions in bradykinin-induced active t-PA release (reduced by 37%, $P=0.03$) and had a marked impairment of SFLLRN-induced t-PA antigen ($P=0.02$) and activity ($P=0.006$) release, with a 96% reduction in overall net t-PA antigen release. SFLLRN also caused similar (P=NS) increases in inactive PAI-1 in both cigarette smokers and non-smokers ($P\leq0.002$ for both). We conclude that cigarette smoking causes marked impairment of PAR-1 mediated endothelial vasomotor and fibrinolytic function. Relative arterial stasis and near abolition of t-PA release will strongly promote clot propagation and vessel occlusion. The impairment of these protective homeostatic

responses suggests a major contribution of impaired endothelial PAR-1 action to the increased incidence of myocardial infarction and stroke in cigarette smokers.

6.2 INTRODUCTION

Smoking tobacco remains one of the most important and consistent modifiable risk factors for myocardial infarction and fatal coronary artery disease [Ambrose and Barua, 2004]. The INTERHEART study revealed that smoking tobacco increases the risk of non-fatal myocardial infarction by up to 7-fold [Teo *et al*, 2006]. The pathophysiological mechanisms underlying this association are likely to be a combination of accelerated atherosclerosis [Zieske *et al*, 2005] and a propensity to acute coronary thrombosis [Burke *et al*, 1997; Ambrose and Barua, 2004].

The endogenous fibrinolytic system is responsible for the dissolution of arterial thrombi that are frequently found on the surface of atherosclerotic plaques at areas of endothelial denudation [Davies *et al*, 1988; Oliver *et al*, 2006]. It is regulated by the pro-fibrinolytic factor, t-PA, and its endogenous inhibitor, PAI-1 [Jansson *et al*, 1993; Meade *et al*, 1993; Nordenhem *et al*, 2005]. The rapid mobilisation of t-PA from the endothelium is crucial, with thrombus dissolution being much more effective if t-PA is incorporated during, rather than after, thrombus formation [Fox *et al*, 1985]. Indeed, acute stimulated t-PA release predicts the future risk of cardiovascular events [Robinson *et al*, 2007].

Thrombin plays a central role in the coagulation cascade and thrombosis. It is one of the most powerful physiological agonists in the cardiovascular system and its actions are

fundamental to the processes of atherothrombosis. Distinct from its enzymatic role in the coagulation cascade, thrombin causes direct cellular activation through stimulation of a novel family of G-protein coupled receptors, protease-activated receptors [Coughlin, 2000]. These receptors have a unique mechanism of activation whereby agonist-induced proteolytic cleavage of the extracellular domain reveals a short peptide sequence that remains tethered and causes auto-activation of the receptor. To date, four different types of PARs have been identified: PAR-1, -3 and -4 are all activated by thrombin whilst PAR-2 is mainly activated by trypsin [Hollenberg and Compton, 2002].

Protease-activated receptor-1 is the principal receptor that mediates the cardiovascular actions of thrombin. The hexapeptide, SFLLRN, represents the short peptide sequence revealed during PAR-1 activation and can be used as a selective agonist of the human PAR-1 thrombin receptor without activation of the coagulation cascade. Using SFLLRN, we have previously described the *in vivo* effects of PAR-1 activation in platelets, endothelium and vascular smooth muscle in man. For the first time, it was demonstrated that thrombin has unique and contrasting effects in the human vasculature including arterial dilatation, venous constriction, platelet activation and t-PA release [Guðmundsdóttir *et al*, 2006].

We, and others, have previously reported that pharmacological stimulation of acute t-PA release in the peripheral [Newby *et al*, 1999; Pretorius *et al*, 2002] and coronary [Newby *et al*, 2001; Takashima *et al*, 2007] arterial circulations is markedly attenuated in smokers. In this study the hypothesis is that smokers have impaired PAR-1 mediated vascular responses. We have therefore, examined PAR-1 mediated t-PA release and

vasomotor responses in the forearm circulation of cigarette smokers and healthy non-smoking control subjects.

6.3 METHODS

6.3.1 SUBJECTS

Twelve healthy cigarette smokers (5-20 cigarettes/day) and twelve age- and sex-matched non-smokers between 20 and 46 years old participated in the study, which was undertaken with the approval of the Lothian Research Ethics Committee and in accordance with the Declaration of Helsinki. The written informed consent of each subject was obtained before entry into the study. Exclusion criteria included a history of asthma, hypertension, diabetes mellitus, coagulopathy, hyperlipidaemia or vascular disease. Control subjects were lifelong non-smokers and were not exposed to regular environmental tobacco smoke. Smokers had a history of regular daily cigarette smoking of at least 5 years and maintained their normal smoking habits in the week before attendance.

None of the subjects received vasoactive or nonsteroidal anti-inflammatory drugs in the week before the study, and all abstained from alcohol for 24 hours before and from food, tobacco, and caffeine-containing drinks on the day of the study. All studies were performed in a quiet, temperature controlled room maintained at 22-24°C.

6.3.2 DRUGS

Custom synthesised clinical grade SFLLRN and bradykinin were purchased from Clinalfa, Laufelfingen, Switzerland. Sodium nitroprusside from David Bull Laboratories, Faulding, UK, and tirofiban from Merck, Sharp and Dohme, Hoddesdon, UK. Infused drugs were all administered following dissolution in 0.9% saline. All solutions were freshly prepared on the day of the study.

6.3.3 STUDY DESIGN

All subjects underwent brachial artery cannulation with a 27 standard-wire-gauge steel needle. The intra-arterial infusion rate was kept constant at 1 mL/min throughout all studies. Forearm blood flow was measured in the infused and non-infused arms by venous occlusion plethysmography using mercury-in-silastic strain gauges as described previously [Newby *et al*, 1997a; Newby *et al*, 1999]. Supine heart rate and blood pressure were monitored at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer.

After a 20-minute intra-arterial infusion of 0.9% saline, the GPIIb/IIIa antagonist, tirofiban (1.25 μg/min), was infused and continued throughout the study to inhibit potential PAR-1 induced platelet aggregation. This dose of tirofiban does not affect forearm blood flow [Guðmundsdóttir *et al*, 2006].

During tirofiban administration, subjects received intra-arterial infusions of the PAR-1 activating peptide, SFLLRN (5, 15 and 50 nmol/min), bradykinin (an endothelium-dependent vasodilator that causes the release of t-PA; 100, 300 and 1000 pmol/min) and

sodium nitroprusside (an endothelium-independent vasodilator that does not release t-PA; 2, 4 and 8 μg/min). Study drugs were infused in random order for 10 minutes at each dose and were separated by a 20-minute infusion of 0.9% saline.

Blood sampling

Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms. Blood samples were drawn simultaneously from each arm at the beginning of the study and during infusion of each dose of PAR-1 activating peptide (SFLLRN), bradykinin and sodium nitroprusside. Venous blood was collected into acidified buffered citrate (Stabilyte, Trinity Biotech Plc, Co. Wicklow, Ireland; for t-PA assays) and into citrate (Becton and Dickinson Vacutainer, BD UK Ltd, Oxford, UK; for PAI-1 and vWF assays). Samples were kept on ice before centrifugation at 2000 *g* for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at 80°C before assay. Plasma t-PA antigen and activity (t-PA Combi Actibind Elisa Kit; Technoclone, Vienna, Austria), PAI-1 antigen and activity (Elitest PAI-1 antigen and Zymutest PAI-1 Activity; Hyphen Biomed, Neuville-Sur-Oise, France) and vWF antigen (Dako A/S, Glostrup, Denmark) concentrations were determined by enzyme-linked immunosorbant assays. Full blood count and haematocrit were measured at baseline and the end of the study.

6.3.4 DATA ANALYSIS AND STATISTICS

Forearm plethysmographic data were analysed as described previously [Newby *et al*, 1997a]. Estimated net release of plasma t-PA, PA-1 and vWF has been defined previously as the product of the infused forearm plasma flow (based on the mean

haematocrit and the infused forearm blood flow) and the concentration difference between the infused and non-infused arms [Newby *et al*, 1997b]. Variables are reported as mean±SEM and analysed using repeated measures ANOVA with post-hoc Bonferroni corrections and two-tailed Students *t*-test as appropriate. Statistical analysis was performed with GraphPad Prism (Graph Pad Software) and statistical significance taken at the 5% level.

6.4 RESULTS

There were no differences in baseline characteristics between cigarette smokers and non-smokers (Table 6.1). There were no changes in blood pressure, heart rate or haematocrit (data not shown) during the study. Smokers had a mean cigarette consumption of 15±1 cigarettes per day over a mean period of 9±2 years (7±2 pack years).

6.4.1 FOREARM BLOOD FLOW

Tirofiban did not affect forearm blood flow (data not shown). Intra-arterial sodium nitroprusside, bradykinin and the PAR-1 activating peptide, SFLLRN, all caused dose-dependent vasodilatation in the infused arm of smokers and non-smokers ($P<0.0001$ for all; ANOVA). There were no changes in blood flow in the non-infused arm (data not shown).

TABLE 6.1 Baseline subject characteristics

	Non-smokers	Smokers
Age (y)	26±2	29±2
Sex (M/F)	12/0	12/0
Body mass index (kg/m^2)	24±1	27±1
Mean arterial pressure (mm Hg)	97±2	99±2
Heart rate (bpm)	66±2	64±2
Baseline haematocrit	0.42±0.01	0.42±0.01

Although there was no difference with bradykinin (P=0.64; ANOVA smokers *versus* non-smokers), vasodilatation to SFLLRN was attenuated in smokers (P=0.044; ANOVA smokers *versus* non-smokers). Endothelium-independent vasodilatation evoked by sodium nitroprusside was similar in both groups (P=0.74; ANOVA smokers *versus* non-smokers; Figure 6.1).

6.4.2 PLASMA FIBRINOLYTIC AND HAEMOSTATIC FACTORS

Baseline plasma t-PA antigen and activity (Table 6.2) and vWF antigen (Table 6.3) concentrations were similar in smokers and non-smokers. There appeared to be a trend towards higher absolute plasma PAI-1 antigen and activity concentrations in smokers but this difference did not reach statistical significance (smokers *versus* non-smokers: PAI-1 antigen, P=0.07 and P=0.10, and PAI-1 activity, P=0.18 and P=0.24; infused and non-infused arms respectively; Table 6.4).

SFLLRN caused a dose-dependent net release of t-PA antigen in non-smokers (P<0.0005; ANOVA) but not smokers (P=0.18; ANOVA; Figure 6.2). In comparison to non-smokers, the release of t-PA antigen and activity by SFLLRN was markedly attenuated in smokers (P=0.02 and P=0.006, respectively; ANOVA). However, SFLLRN induced a dose-dependent net release of PAI-1 antigen release in both non-smokers (P=0.0002; ANOVA) and smokers (P=0.001; ANOVA). The response was similar in both groups (P=0.36; ANOVA) and was associated with no change in net PAI-1 activity (P=NS for all; ANOVA [data not shown]) or vWF antigen release (P=NS; ANOVA; Table 6.3).

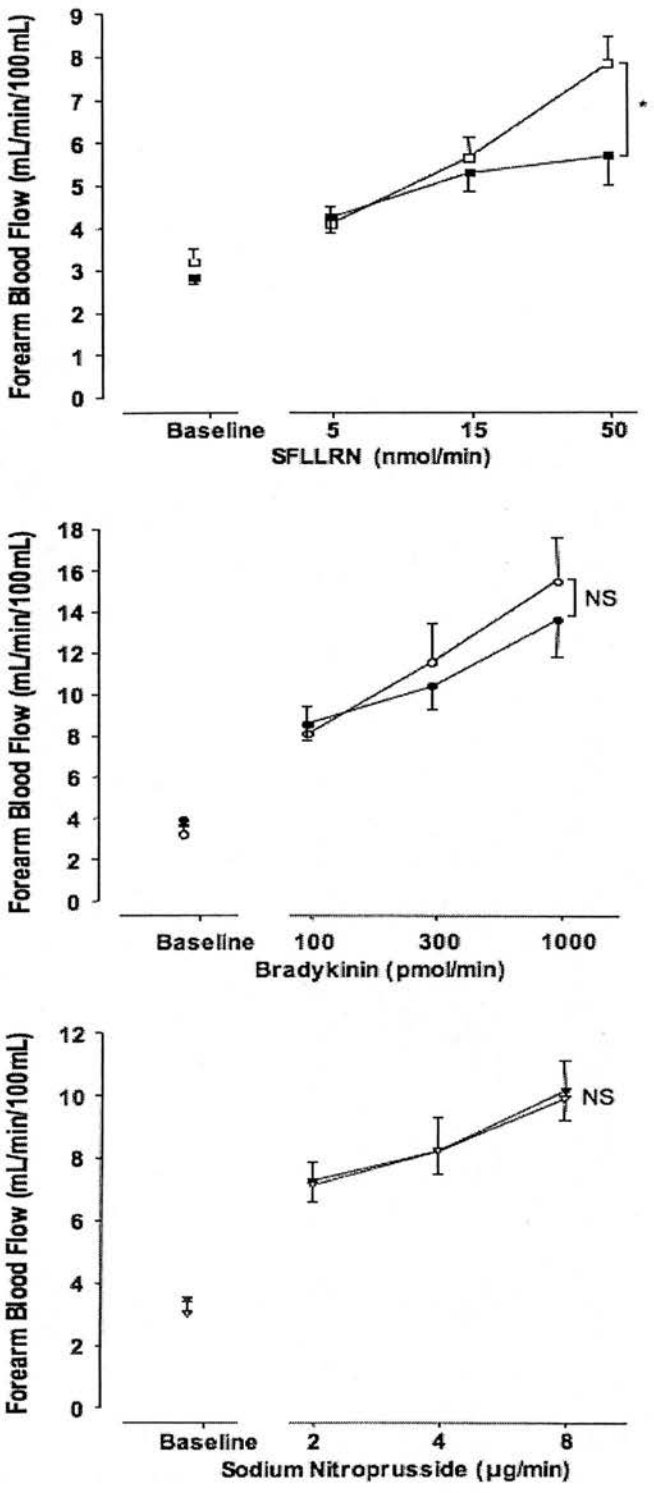

Figure 6.1 Forearm arterial vasodilatation in smokers and non-smokers. Forearm arterial vasodilatation induced by SFLLRN (squares), bradykinin (circles) sodium nitroprusside (triangles) in smokers (closed symbols) and non-smokers (open symbols). *P<0.05, NS=non-significant; analysis of variance (ANOVA), smokers *versus* non-smokers.

TABLE 6.2 Absolute plasma t-PA antigen and activity concentrations

	t-PA ANTIGEN (ng/mL				t-PA ACTIVITY (IU/mL)			
	Non-smokers		Smokers		Non-smokers		Smokers	
Arm	**Infused**	**Non-Infused**	**Infused**	**Non-Infused**	**Infused**	**Non-Infused**	**Infused Arm**	**Non-Infused**
Baseline	8.15±2.18	8.33±2.24	10.83±1.94	10.93±2.23	0.48±0.08	0.45±0.06	0.36±0.07	0.35±0.07
Pre SFLLRN	8.08±2.07	8.41±1.92	11.35±2.2	11.15±2.29	0.54±0.08	0.44±0.05	0.41±0.07	0.40±0.07
SFLLRN 5 nmol/min	7.84±2.23	7.68±1.76	9.91±2.08	11.51±2.70	0.63±0.07	0.51±0.05	0.44±0.08	0.44±0.08
SFLLRN 15 nmol/min	7.46±2.08	7.74±2.00	10.56±1.85	10.83±2.52	0.84±0.11	0.59±0.06	0.56±0.11	0.50±0.08
SFLLRN 50 nmol/min	11.54±2.82†	6.93±1.87	12.08±2.58	11.17±2.73	2.01±0.38†	0.65±0.09	0.78±0.18*§	0.49±0.10
Pre BK	7.14±1.68	8.17±2.43	10.66±1.99	10.54±2.12	0.55±0.06	0.53±0.07	0.35±0.08	0.40±0.08
BK 100 pmol/min	9.39±1.76	7.55±2.19	12.82±2.76	10.44±2.11	1.55±0.21	0.54±0.07	0.84±0.15	0.45±0.07
BK 300 pmol/min	9.32±1.55	8.23±2.42	13.66±2.66	12.42±2.78	2.15±0.31	0.74±0.10	1.60±0.29	0.57±0.12
BK 1000 pmol/min	11.55±1.77†	8.44±1.57	17.82±3.49†	12.48±2.70	3.51±0.40†	0.97±0.12	2.25±0.45†§	0.73±0.13‡

One-way analysis of variance (ANOVA) for dose response. *P<0.01, †P<0.005; two-way ANOVA, ‡P<0.05, §P<0.001 smokers *versus* non-smokers.
Tissue plasminogen activator - t-PA; Bradykinin - BK.

TABLE 6.3 Plasma von Willebrand factor (vWF) concentrations during SFLLRN and bradykinin infusions

	vWF ANTIGEN (ng/mL)			
	Non-smokers		**Smokers**	
Arm	**Infused**	**Non-Infused**	**Infused**	**Non-Infused**
Baseline	0.72±0.05	0.75±0.19	0.77±0.09	0.78±0.13
Pre- SFLLRN	0.71±0.06	0.80±0.06	0.79±0.07	0.71±0.09
SFLLRN 5 nmol/min	0.70±0.03	0.74±0.05	0.72±0.10	0.73±0.06
SFLLRN 15 nmol/min	0.64±0.03	0.73±0.03	0.78±0.06	0.63±0.06
SFLLRN 50 nmol/min	0.70±0.04	0.74±0.04	0.78±0.06	0.63±0.06
Pre BK	0.74±0.06	0.74±0.05	0.84±0.10	0.74±0.08
BK 100 pmol/min	0.76±0.07	0.73±0.05	0.78±0.08	0.71±0.05
BK 300 pmol/min	0.76±0.06	0.74±0.05	0.83±0.08	0.68±0.08
BK 1000 pmol/min	0.78±0.06	0.76±0.05	0.83±0.08	0.76±0.07

One-way analysis of variance (ANOVA) for dose response, P=NS for all; two-way ANOVA, P=NS for all, smokers *versus* non-smokers.
Bradykinin - BK; Non-significiant - NS.

TABLE 6.4 Absolute plasma PAI-1 antigen and activity concentrations

	PAI-1 ANTIGEN (ng/mL)				PAI-1 ACTIVITY (AU/mL)			
	Non-smokers		Smokers		Non-smokers		Smokers	
Arm	**Infused**	**Non-Infused**	**Infused**	**Non-Infused**	**Infused**	**Non-Infused**	**Infused Arm**	**Non-Infused**
Baseline	23.58±3.93	23.46±3.71	40.09±8.13	34.56±5.50	0.80±0.15	0.86±0.15	1.48±0.48	1.55±0.60
Pre SFLLRN	21.25±3.35	21.68±3.12	33.9±7.19	33.08±5.06	0.63±0.12	0.73±0.13	1.52±0.56	1.57±0.63
SFLLRN 5 nmol/min	21.03±3.23	21.81±3.36	38.52±10.65	32.03±6.04	0.62±0.12	0.71±0.14	1.52±0.56	1.80±0.61
SFLLRN 15 nmol/min	22.28±3.55	21.13±3.18	37.63±9.06	32.92±6.07	0.58±0.12	0.72±0.13	1.65±0.53	1.63±0.63
SFLLRN 50 nmol/min	31.56±4.33*	20.52±3.20	53.64±10.20†	34.97±5.86	0.55±0.13	0.72±0.14	1.59±0.50	1.60±0.67
Pre BK	24.16±3.86	21.58±3.27	38.44±9.67	37.41±8.37	0.79±0.17	0.81±0.16	1.74±0.57	1.70±0.69
BK 100 pmol/min	24.83±4.30	21.83±3.13	38.13±9.93	37.44±7.90	0.66±0.18	0.77±0.17	1.54±0.53	1.78±0.75
BK 300 pmol/min	22.13±3.24	23.68±4.17	39.42±0.39	37.10±8.23	0.56±0.16	0.76±0.19	1.58±0.56	1.35±0.47
BK 1000 pmol/min	20.57±3.20	20.62±2.91	34.53±8.63	32.11±6.57	0.45±0.13	0.62±0.16	1.33±0.50	1.19±0.40

One-way analaysis of variance (ANOVA) for dose response. *P<0.05, †P<0.0005; two-way ANOVA, P=NS for all, smokers *versus* non-smokers.
Plasminogen activator inhibitor type 1 - PAI-1; Bradykinin - BK; Non-significant - NS.

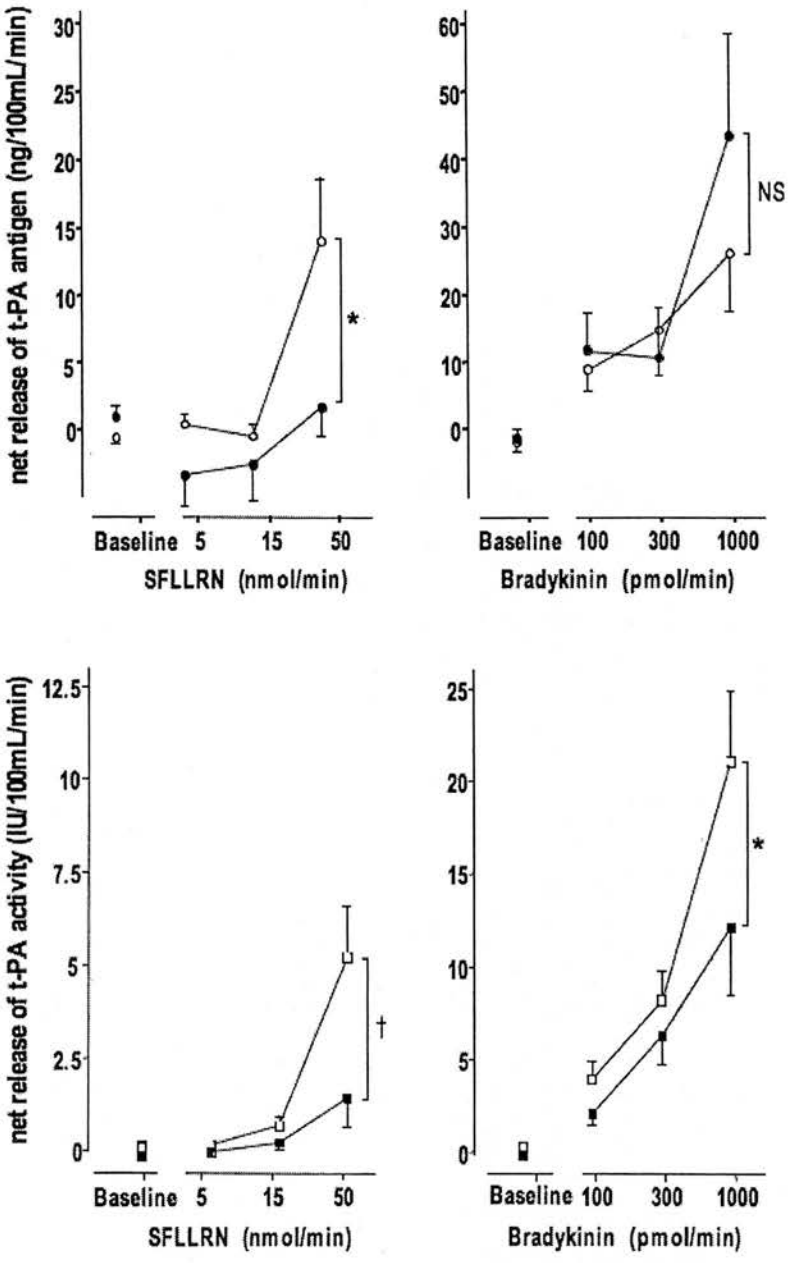

Figure 6.2 Net release of tissue plasminogen activator (t-PA) in smokers and non-smokers. Net t-PA antigen (circles) and activity (squares) release induced by SFLLRN (top panels) and bradykinin (bottom panels) in smokers (closed symbols) and non-smokers (open symbols). *P<0.05, †P=0.005, NS=non-significant; analysis of variance (ANOVA), smokers *versus* non-smokers.

Bradykinin caused a dose-dependent net release of t-PA antigen and activity in both smokers and non-smokers ($P<0.01$ for all; ANOVA). Bradykinin also evoked a dose-dependent increase in absolute t-PA activity in the non-infused arm of both non-smokers ($P<0.0001$; ANOVA) and smokers ($P=0.008$; ANOVA). Net release of t-PA activity induced by bradykinin was less in smokers than non-smokers ($P=0.032$, smokers *versus* non-smokers; ANOVA; Figure 6.2). Bradykinin caused no change in net PAI-1 antigen or activity and did not affect vWF antigen in either group ($P=0.91$, non-smokers; $P=0.98$, non-smokers; ANOVA). As expected [Brown *et al*, 1999] sodium nitroprusside caused no change in absolute or net release of t-PA, PAI-1 or vWf (data not shown).

6.5 DISCUSSION

Here it has been demonstrated that thrombin mediated vascular responses are markedly impaired in cigarette smokers with a substantial reduction in PAR-1 mediated endothelial t-PA release and forearm arterial vasodilatation. This impaired vasomotor and fibrinolytic response may represent an important shift in the fine balance between intravascular thrombosis and fibrinolysis that could account for the increased incidence of atherothrombosis in cigarette smokers.

6.5.1 SMOKING AND PAR-1 INDUCED ARTERIAL VASOMOTION

As reported by others [Pretorius *et al*, 2002], no effect of smoking status on endothelium-dependent vasodilatation to bradykinin or endothelium-independent vasodilatation to sodium nitroprusside was observed. One of the important novel

observations from this study is that vasodilatation evoked via PAR-1 is impaired in smokers, especially at the higher doses of SFLLRN. Because homeostatic mechanisms attempt to maintain vessel patency and minimise intravascular thrombus formation in healthy arteries, we have previously hypothesised that the arterial vasodilatation to PAR-1 activation represents a protective feedback mechanism. In the presence of a developing thrombus, PAR-1 mediated vasodilatation will increase blood flow to limit arterial thrombosis by facilitating its rapid clearance and dissolution [Guðmundsdóttir *et al*, 2006]. Thus, this specific impairment of PAR-1 induced vasodilatation may have major pathophysiological consequences during acute thrombotic events such as myocardial infarction.

6.5.2 SMOKING AND PAR-1 INDUCED RELEASE OF ENDOTHELIUM-DERIVED FACTORS

Over and above diminished vasomotion, the major finding of this study was the almost complete abolition of PAR-1 mediated t-PA antigen release in cigarette smokers. Furthermore, PAR-1 activation caused only a very modest rise in t-PA activity despite causing substantial t-PA antigen and activity release in non-smokers.

The current findings confirm previous studies from our own and other groups reporting reduced t-PA release in cigarette smokers [Newby *et al*, 1999; Pretorius *et al*, 2002; Takashima *et al*, 2007]. Although not seen with t-PA antigen, the present finding of reduced bradykinin-induced active t-PA release is consistent with similar observations previously reported by Pretorius *et al* [2002]. However, the magnitude of the reduction in t-PA release is substantially greater for PAR-1 evoked responses than it is for bradykinin or substance P (96% *versus* 40-50%) [Newby *et al*, 1999;

Pretorius *et al*, 2002]. It is therefore possible that SFLLRN evoked t-PA release may be a more sensitive and pathophysiologically relevant assessment of endothelial vasomotor and fibrinolytic function.

Of note, PAR-1 activation also caused the release of PAI-1 antigen but did not cause an appreciable rise in PAI-1 activity and neither indices were altered by smoking status. This rise in PAI-1 antigen without a change in activity suggests that SFLLRN is releasing PAI-1 from platelets rather than the endothelium since platelet-derived PAI-1 is relatively inactive due to the absence of the stabilising effects of vitronectin [Seiffert *et al*, 1994; Pretorius *et al*, 2002]. Furthermore, our recent work has demonstrated a concomitant rise in β-thromboglobulin suggesting degranulation of platelet alpha granules [Guðmundsdóttir *et al*, 2008]. Therefore, the contribution of the endogenous fibrinolytic system to the prothrombotic state found in cigarette smokers is likely to be driven by impaired endothelial t-PA release and not by alterations in PAI-1 release or activity.

6.5.3 PAR-1 ACTIVATION AS A PATHOPHYSIOLOGICALLY RELEVANT MARKER OF ENDOTHELIAL FUNCTION

Previous studies to assess the endothelial release of endogenous fibrinolytic factors have employed diverse methods. Historical means of stimulating t-PA release have included systemic intravenous infusion of desmopressin and bradykinin but this causes significant confounding effects by altering systemic haemodynamics, activation of the sympathetic nervous system and concomitant release of other mediators [Oliver *et al*,

2005]. By assessing the regional release of t-PA and PAI-1 in response to locally acting agonists, such confounding effects are avoided.

Our group has previously demonstrated that substance P-induced t-PA release in the peripheral [Newby *et al*, 1999] and coronary [Newby *et al*, 2001] circulations is impaired in cigarette smokers, and predicts future adverse cardiovascular events in patients with coronary heart disease [Robinson *et al*, 2007]. However, whilst substance P has been a useful pharmacologic tool, it is unclear whether substance P is likely to act as a major pathophysiological mediator in atherothrombosis. In contrast, bradykinin may have a more direct role since it is released during the contact phase of coagulation and there is enhanced activation of the kallikrein system and bradykinin release in patients with acute coronary syndromes [Hoffmeister *et al*, 1995]. However, it can be argued that, given its central role in thrombosis and inflammation, thrombin is the most powerful and pathophysiologically relevant mediator in this setting. These present findings not only reinforce previous findings but give the clearest indication yet that impaired endothelial function is of critical and dynamic importance in the setting of coronary heart disease and acute coronary syndromes.

6.5.4 Smoking and endothelium-dependent mechanisms

Protease-activated receptor-1 appears to mediate arterial vasodilatation via two endothelium-dependent mechanisms, NO and EDHF [Guðmundsdóttir *et al*, 2008]. The pathways via which PAR-1 activation causes the endothelial release of t-PA are less clear and, in fact, inhibition of NO synthesis causes augmented SFLLRN-induced t-PA release [Guðmundsdóttir *et al*, 2008]. This has raised the question as to whether EDHF

is responsible for t-PA release and, in the absence of NO, EDHF responses undergo a compensatory up-regulation. Whilst the bulk of evidence suggests that smoking predominantly affects endothelial function by increasing oxidative stress with consequent disruption of NO production [Kiowski *et al*, 1994; Puranik and Celermajer, 2003], studies specifically examining the effect of smoking upon EDHF mediated responses are lacking.

6.5.5 Study limitations

The forearm circulation has been an extremely reliable model for the assessment of vascular physiology and pathophysiology. We do accept that the findings in the forearm may not be accurately representative of the coronary circulation. However, we and others have previously demonstrated consistent findings of impaired endothelial t-PA release in both the forearm [Newby *et al*, 1999; Pretorius *et al*, 2002] and coronary [Newby *et al*, 2001; Takashima *et al*, 2007] circulations of cigarette smokers. Although the forearm vascular bed is relatively protected from the development of atheroma, it therefore seems likely that changes in its fibrinolytic capacity are indicative of the coronary circulation.

It could be argued that the safety requirement for the co-administration of tirofiban with SFLLRN detracts from its advantage as a pathophysiologically relevant agent for the assessment of endothelial function. However, this locally active dose of GPIIb/IIIa inhibitor abolishes SFLLRN mediated platelet aggregation without affecting platelet-monocyte aggregation, a sensitive marker of platelet activation. Furthermore, it has no effect upon forearm blood flow or fibrinolytic responses to SFLLRN

[Guðmundsdóttir *et al*, 2006]. We therefore believe that SFLLRN remains an important and relevant tool. However, we accept that hitherto unrecognised and subtle vascular effects of tirofiban may introduce a degree of confounding to the interpretation of SFLLRN-induced responses.

We have demonstrated an important impairment of fibrinolytic capacity in smokers but it remains unclear whether this reflects an impairment of synthesis, storage and release of t-PA, or indeed acceleration of its degradation. Addressing these questions will be challenging and is likely to require specifically designed *in vitro* studies.

6.5.6 Conclusions

In healthy vessels, thrombin's powerful procoagulant and prothrombotic effects are offset by its ability to evoke the release of t-PA and induce arterial vasodilatation. We have shown here that cigarette smoking causes a marked impairment in PAR-1 mediated endothelial vasomotor and fibrinolytic function. Relative arterial stasis and abolition of t-PA release will strongly enhance clot expansion and vessel occlusion. Taken together, these findings suggest a major contribution of impaired endothelial PAR-1 action to the increased atherothrombotic risk of smokers. These important and novel findings are of direct relevance to our understanding of the pathophysiology by which cigarette smoking causes an increased propensity to atherothrombotic disorders including acute myocardial infarction and stroke.

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 PAR ACTIVATION OF HUMAN PLATELETS

7.1.1 *IN VITRO* EFFECTS OF PAR ACTIVATION IN HUMAN PLATELETS

Thrombin is one of the most important physiological stimulants of platelet activation that in human platelets, appears to be mediated largely through the PAR-1 receptor, as well as by PAR-4 and GPIb receptors [Kahn *et al*, 1999; Lova *et al*, 2004], whereas PAR-2 and PAR-3 are not expressed on human platelets. Prior to studies in the vasculature, the effects of PAR-1 activation on human platelet aggregation and activation *in vitro* were explored. The PAR-1 activating peptide SFLLRN, with and without a PAR-1 antagonist, was compared with other platelet agonists such as thrombin and the thromboxane A_2 agonist U46619. Although PAR-1 activation has been addressed extensively in human and animal platelet studies in the past, it was important to obtain concentration-response curves for both our research and clinical grade PAR activating peptides prior to assessing the effects of PAR activation *in vivo* and we have confirmed that SFLLRN, thrombin and U46619 all caused concentration-dependent platelet aggregation.

Selective PAR-1 antagonists (thrombin receptor antagonists) have recently been developed as antiplatelet agents. Protease-activated receptor-1 antagonists block thrombin receptors without affecting the enzymatic cleavage of thrombin in the coagulation cascade, and therefore inhibit direct cellular effects of thrombin but not fibrin production. There are currently ongoing large phase III clinical trials assessing the thrombin receptor antagonist SCH530348 [Angiolillo and Guzman, 2008; Chintala *et al*, 2008], but this drug has not been readily available for academic

research. We have assessed the *in vitro* effects of the PAR-1 antagonist RWJ-58259 and confirmed that it fully inhibits SFLLRN-induced platelet aggregation but did not affect aggregation induced by U46619.

Thrombin-induced platelet aggregation was only partially inhibited by the PAR-1 antagonist RWJ-58259 and this was not augmented with increasing concentrations of the antagonist nor reduced by increasing concentrations of thrombin. Thrombin-induced platelet aggregation was however, as expected, fully blocked by the direct thrombin inhibitor lepirudin. The findings with lepirudin and RWJ-58259 suggest the presence of an alternative platelet receptor for thrombin when PAR-1 receptors are blocked. PAR-2 and PAR-3 receptors are not present on human platelets. In our studies the PAR-4 activating peptide GYPGKV did not induce human platelet aggregation at concentrations up to 3 mM. PAR-4 is a very low affinity receptor and PAR-4 activation is unlikely to solely account for the PAR-1 independent platelet aggregation induced by thrombin. The GPIb receptor is also known to be stimulated by thrombin and may be a more likely candidate [Soslau *et al*, 2001].

Activation of platelets by thrombin induces calcium-dependent expression of P-selectin on the platelet surface and subsequent binding to monocytes through the counter-receptor P-selectin glycoprotein ligand-1 [Jungi *et al*, 1986; Sako *et al*, 1993]. Platelet activation was assessed by measuring levels of platelet-monocyte binding in whole blood with flowcytometry. Concentration-response curves were obtained for PAR-1 agonism with and without GPIIb/IIIa inhibition with tirofiban and compared with platelet aggregometry findings. Protease-activated receptor-1

agonism continued to induce platelet activation as measured by levels of platelet-monocyte binding in the presence of tirofiban, whereas tirofiban completely inhibited PAR-1 induced platelet aggregation measured by platelet aggregometry. This is consistent with previous findings that although GPIIb/IIIa receptor antagonism inhibits the final steps leading to platelet aggregation, it does not prevent platelet activation and platelet-monocyte aggregate formation [Sarma *et al*, 2002].

7.1.2 PAR-1 STIMULATION AND PLATELET ACTIVATION *IN VIVO*

We have demonstrated for the first time that PAR-1 agonism with intra-arterial SFLLRN caused very marked platelet activation *in vivo* as measured by platelet-monocyte binding (from baseline of 16±5% to peak of 74±5%), but declined towards baseline with time. The half-life of the platelet-monocyte aggregates appeared to be around 1 hour and this would be in keeping with the aggregation being irreversible and the half-life being determined by clearance from the circulation (see section 7.2).

By infusing tirofiban before and during SFLLRN it was possible to measure increased levels of platelet activation during PAR-1 stimulation *in vivo*, whilst preventing platelet aggregation and potential thrombosis. Tirofiban infusion on its own did not affect baseline platelet activation.

7.2 THE VASCULAR EFFECTS OF PAR ACTIVATION

Establishing the receptor mediated effects of thrombin in the vasculature is of major physiological and therapeutic relevance and the direct vascular effects of thrombin

receptor agonism have not been previously reported in man. The aim of these studies was to assess the role of PAR-1 receptors in the human vasculature. Investigation of the *in vivo* vascular effects of thrombin is challenging as direct activation of the coagulation cascade and platelets could result in intravascular thrombosis and tissue damage. The PAR-1 activating peptide SFLLRN was therefore used to achieve direct cellular stimulation through thrombin receptors without enzymatic activation of the coagulation cascade.

Thrombin and PAR-1 activating peptides have previously been shown by others to cause diverse vasomotor effects in myography studies on *ex-vivo* vascular segments. In canine and porcine ring segments, thrombin and PAR-1 activating peptide cause endothelium- and NO-dependent venous and arterial dilatation [Ku and Zaleski, 1993 Tesfamariam *et al*, 1993]. In human tissue thrombin causes endothelium-dependent vasodilatation in internal mammary and coronary artery ring segments [Yang *et al*, 1994], whereas in human umbilical and placental arteries thrombin and PAR-1 agonism have different effects causing an endothelium-independent contractile response, and no endothelium mediated relaxing response [Tay-Uyboco *et al*, 1995]. Whilst to some extent myography studies can be difficult to interpret, this is also likely to reflect differences in PAR expression and translation of PAR signalling in different vascular beds [Fujiwara *et al*, 2004].

Activation of PAR-1 and PAR-2 *in vivo* in mice causes vasodilatation, where the hypotensive effect of PAR-1 but not PAR-2 is reversed by inhibition of NO leading to vasoconstriction [Cheung *et al*, 1998]. In rabbits, femoral artery balloon injury has

been shown to cause up-regulation of PAR-1 expression and to induce a vasoconstrictive response to PAR-1 activation [Fukunaga *et al*, 2006].

The marked species variability in PAR expression necessitates the direct exploration of PAR function in man since animal models are likely to be unrepresentative of the clinical setting. This project was further challenged by the absence of specific non-human primate data on the effects of PAR-1 agonism. The clinical studies were therefore planned in a cautious incremental fashion. The first step was to carry out preliminary *in vitro* studies to assess the effects on platelets and to obtain concentration-response curves, followed by pilot studies *in vivo* using very low concentrations of the agonists. Initially the effects of PAR-1 agonism were assessed in the dorsal hand vein, where very low doses of drugs can be used to measure localised venomotor responses. We then moved on to the arterial circulation using the forearm model, first in healthy volunteers looking at both effects and mechanism and subsequently comparing smokers with non-smokers. In the arterial studies tirofiban was co-infused throughout to prevent platelet aggregation.

7.2.1 VENOUS EFFECTS OF PAR ACTIVATING PEPTIDES

Having confirmed previous observations of PAR-2 mediated venodilatation [Robin *et al*, 2003], the discovery that PAR-1 caused dose-dependent venoconstriction was unexpected. Initial studies were carried out after inducing venous tone with norepinephrine, to be able to detect either constriction or dilatation as veins do not have a resting tone. Further studies were carried out to look at this effect in more detail, and PAR-1 activation caused consistent marked venoconstriction both with

and without norepinephrine or tirofiban co-infusion, as well as after endothelial denudation where absence of functional endothelium had been confirmed. In combination with the apparent absence of *in situ* thrombosis, this suggests that PAR-1 induced venoconstriction was not mediated by platelet aggregation although an effect of platelet activation can not be precluded. We therefore believe that this potent venoconstriction is most likely due to direct effect on vascular smooth muscle.

7.2.2 ARTERIAL EFFECTS OF PAR ACTIVATING PEPTIDES

In contrast to the venous effects, both PAR-1 and PAR-2 activation caused dose-dependent vasodilatation of forearm resistance vessels, with PAR-2 having a much smaller effect and requiring much higher doses.

Protease-activated receptor expression varies between endothelial cell cultures originating from different human blood vessels [Fujiwara *et al,* 2004] and this may partly explain the different responses between PAR-1 and PAR-2 in the arterial and venous circulations. Our findings that PAR-2 activation causes venous and arterial dilatation are in keeping with previous findings [Robin *et al*, 2003] and consistent with the vascular role of PAR-2 receptors in inflammatory conditions.

The vasomotor effects of PAR-1 activation were short-acting, with forearm blood flow returning to baseline during a 6-minute washout infusion. The effect on levels of platelet-monocyte binding was longer lasting, with a half-life of approximately 1 hour. This is in keeping with *in vitro* studies in various cell lines that have demonstrated that PAR-1 receptors are rapidly internalised by endocytosis after

activation, terminating the effect. A sustained signal therefore requires mobilisation of intact receptors from intracellular stores or the synthesis of new receptors [Trejo *et al*, 2000]. Platelets on the other hand only need to respond once. Platelets do not internalise their receptors, and neither have the ability to synthesise new receptors, nor have large intracellular stores and thrombin causes irreversible platelet aggregation. The half-life of platelet-monocyte aggregates therefore appears to be determined by their clearance from the circulation.

The study protocol was vigilant to minimise any potential prothrombotic effects associated with PAR-1 induced platelet aggregation. Having defined the *in vitro* concentration of tirofiban that would permit direct *in vivo* intravascular infusion of PAR-1 activating peptide, without inducing platelet aggregation and potential thrombosis, tirofiban was infused throughout arterial studies. No significant adverse clinical side-effects or thrombosis were observed, although the generation of subclinical microthrombi can not be fully excluded. It could be argued that, in these studies, the safety requirement for the co-administration of tirofiban with SFLLRN in the arterial studies detracts from our findings. However, locally active doses of tirofiban were used, that abolish SFLLRN mediated platelet aggregation without affecting platelet-monocyte binding, a sensitive marker of platelet activation. The total dose of tirofiban administered (approximately 200 μg over 150 min) is much lower than that required to achieve systemic antiplatelet effects in clinical practice (28 μg/min loading dose for 30 minutes followed by 7 μg/min maintenance dose). Furthermore, at this dose, tirofiban had no effect upon basal forearm blood flow or fibrinolytic or platelet responses to SFLLRN. We therefore believe that SFLLRN is

an important and relevant tool to assess these fundamental pathophysiological aspects of endothelial function.

7.3 THE ROLE OF ENDOTHELIUM IN VASCULAR EFFECTS OF PARs

Animal models indicated that PAR induced vasodilatation was likely to be endothelium- and NO-dependent [Cheung *et al*, 1998)]. However, as previously discussed, data from animal studies have limited relevance in man because of the wide species variability in PAR-1 receptor expression and function. We have described the contrasting role of the endothelium in the vascular actions of PAR-1 *in vivo* in man. Whilst not providing a major contribution to venoconstriction or PAI-1 release, the endothelium mediates PAR-1 induced arterial vasodilatation and t-PA release. Our findings provide clear evidence of a major interaction between the vascular endothelium and thrombin *in vivo* in man. Furthermore, they highlight the critical importance of endothelial function at the time of acute arterial injury and intravascular thrombosis, such as during acute coronary syndromes.

7.3.1 ROLE OF THE ENDOTHELIUM IN PAR-I INDUCED VASOMOTION

Having described the unexpected finding of PAR-1 induced venoconstriction in man we wanted to address whether this effect could be mediated by either a direct action on vascular smooth muscle or via the release of endothelium-derived vasoconstrictors, such as endothelin or angiotensin II. We therefore assessed PAR-1 induced venoconstriction before and after endothelial denudation by instillation of distilled water where the absence of functional endothelium was confirmed by a lack

of venodilatation to acetylcholine. Activation of PAR-1 continued to induce a marked dose-dependent venoconstriction even in the absence of the endothelium, suggesting a dominant and direct effect of PAR-1 on the vascular smooth muscle cells.

In contrast to effects on the venous circulation, PAR-1 agonism causes potent arterial vasodilatation. This suggests a different effect on the arterial vasculature that is likely to be mediated by the endothelium. It would be difficult and not safe to conduct comparable *in vivo* endothelial denudation studies in the arterial circulation. A pharmacological approach to the inhibition of the three main known mediators of endothelium-dependent vasodilatation, prostacyclin, NO and EDHF was therefore used. Although prostacyclin inhibition appeared to have no effect, inhibition of NO and potassium channels both attenuated the PAR-1 induced vasodilatation. Combined inhibition of all three almost abolished the vasodilator actions of PAR-1 agonism. This suggests that, unlike the venous circulation, PAR-1 mediated arterial actions are dominated by, and dependent upon, the vascular endothelium and are mediated by NO and EDHF.

These results are in keeping with previous *ex vivo* myography studies on the vasomotor effects of thrombin and PAR-1 activating peptides on human coronary and internal mammary artery ring segments that suggested that thrombin causes endothelium-dependent vasodilatation [Yang *et al*, 1994]. Our group has also previously shown concordance between the coronary and forearm circulations

[Newby *et al*, 1999; Newby *et al*, 2001] and our findings are likely to reflect the vasomotor response to thrombin receptor activation in the coronary circulation.

7.3.2 PAR-1 INDUCED RELEASE OF ENDOTHELIUM-DERIVED FACTORS

We have demonstrated that as well as having vasomotor effects, PAR-1 has important effects on the release of endothelium-derived coagulant and fibrinolytic factors causing endothelial t-PA release without affecting vWF, which is in keeping with studies of other G-protein coupled receptor vasodilators [Oliver *et al*, 2005]. Looking into the mechanism of t-PA release, SFLLRN-induced t-PA release appeared to be augmented by the inhibition of endogenous NO production. Bradykinin-induced t-PA release has previously been found to be independent of nitric oxide and cyclooxygenase activity, and it has been suggested that EDHF is responsible for its release [Brown *et al*, 2000]. It is possible that when NO synthesis is inhibited, EDHF is up-regulated and accounts for the augmented t-PA release induced by SFLLRN. However, in contrast to tetraethylammonium's inhibitory effects on SFLLRN-induced arterial vasodilatation, it had no effect on SFLLRN evoked t-PA release, suggesting that t-PA release is independent of potassium channels.

A further novel finding in the arterial studies was the increase in PAI-1 release, especially during NO synthase inhibition. There have been no previous reports of acute increases in plasma PAI-1 concentrations following the administration of endothelial agonists, especially using the forearm model [Oliver *et al*, 2005]. Whilst the endothelium is an important source of PAI-1, we believe these findings are

consistent with acute platelet release of PAI-1. There are several reasons to support this. First, although PAI-1 antigen concentrations increased, there was no corresponding rise in PAI-1 activity. Indeed, PAI-1 activity fell during marked release of t-PA. Plasminogen activator inhibitor type 1 is stored in platelet α-granules where its activity is very low (<5% of the activity seen in plasma) due to the absence of the stabilising effect of vitronectin. In contrast, endothelium-derived PAI-1 would be anticipated to remain active. Second, there was no concurrent rise in vWF confirming a selective effect on the endothelium with isolated t-PA release. Third, we have also demonstrated concomitant platelet activation with marked increases in platelet-monocyte aggregate formation and release of β-thromboglobulin; the latter is also stored in the α-granules of platelets. Finally, PAR-1 induced PAI-1 release was augmented during the 'nitric oxide clamp'. Nitric oxide has important antiplatelet effects and, in the presence of its inhibition, increased platelet activation may have led to greater PAI-1 release.

7.4 COMPARISION OF EFFECTS OF PAR ACTIVATION BETWEEN SMOKERS AND NON-SMOKERS

Cigarette smoking is strongly associated with atherothrombotic events such as myocardial infarction and stroke. This is caused by multiple pathogenetic pathways including accelerated atherosclerosis, increased platelet activation and impaired endogenous fibrinolysis. Burke *et al* have previously demonstrated that among men who died of sudden coronary death, smokers have a significantly higher incidence of acute thrombosis, whereas those with hyperlipidaemia are more likely to develop

plaque rupture [Burke *et al*, 1997]. Thrombin is central to this process, probably through its receptor mediated effects via PAR-1, but a direct causative link has not been previously established *in vivo*. We have described a marked impairment of PAR-1 mediated vasodilatation and endogenous fibrinolysis in otherwise healthy, young cigarette smokers *in vivo*. Forearm arterial flow was compared in response to PAR-1 activation, bradykinin and sodium nitroprusside. Whilst all agonists caused similar increase in forearm blood flow, it was only the response to PAR-1 activation that was significantly impaired in smokers. The most striking finding was that endogenous net t-PA release to PAR-1 activation was almost abolished in smokers compared with non-smokers. The impairment of these protective responses may shift the balance between intravascular thrombosis and endogenous fibrinolysis towards thrombotic events and suggests a major contribution of impaired endothelial PAR-1 action to the increased incidence of myocardial infarction and stroke in cigarette smokers.

7.5 SELECTION OF PAR ACTIVATING PEPTIDES

The PAR-1 activating peptide SFLLRN was chosen as PAR-1 agonist for several reasons. The PAR activating peptides have been extensively investigated *in vitro*, both by ligand binding and functional assays and by peptide binding studies.

The hexapeptide SFLLRN is as selective and has the same effect as the fourteen amino acid active cleaved tail of the human PAR-1 receptor [Scarborough *et al*, 1992]. Another hexapeptide, TFLLRN is a synthetic modification of this. We accept

that current data suggest that TFLLRN has greater PAR-1 selectivity than SFLLRN, with PAR-1/PAR-2 selectivity for SFLLRN being approximately 4:1 *versus* 220:1 for TFLLRN [Kawabata *et al*, 1999]. We do however believe that by using SFLLRN, the *in vivo* molecular mechanisms and results of PAR-1 cleavage are more closely represented.

The effects of the PAR-1 activating peptide SFLLRN were compared with the PAR-2 activating peptide SLIGKV, the endothelium-dependent vasodilator bradykinin and the endothelium-independent vasodilator sodium nitroprusside, and not with a scrambled peptide. SLIGKV has previously been compared with a scrambled peptide *in vivo* [Robin *et al*, 2003] using similar vascular methods, and the scrambled peptide had no effect at concentrations about 30-fold of those that we used. Our group has also tested the effects of similarly sized peptides in the past demonstrating no effect.

It is recognised that there can be functional interaction and cross-activation between different PAR receptors. Thrombin activates PAR-1, PAR-3 and PAR-4 at different potencies but not PAR-2 which is mainly activated by trypsin and some coagulation factors. The PAR-1 activating peptide SFLLRN can also activate the PAR-2 receptor but not vice versa [Blackhart *et al*, 1996]. We believe that the vascular effects seen in our studies are a reflection of PAR-1 activation and do not reflect PAR-2 activity. We have assessed the *in vivo* effects of intra-arterial SFLLRN and SLIGKV, the selective PAR-2 agonist. Whilst SFLLRN caused potent arterial vasodilatation, SLIGKV caused only minor arterial vasodilatation at doses approximately 160-fold greater than those of SFLLRN (Figure 4.3). Furthermore, SFLLRN caused marked

t-PA antigen and activity release, whilst PAR-2 agonism had no effect on t-PA levels. They also had opposing effects in the venous circulation, PAR-1 activation with SFLLRN causing venoconstriction whilst PAR-2 activation caused venodilatation. Whilst expression of the PAR-2 receptor may be up-regulated by inflammation, the vascular effects of PAR-2 are likely to be modest in the healthy human *in vivo*. Finally, although the PAR-2 receptor can *in vitro* be activated by SFLLRN at high concentrations [Al Ani *et al*, 1999], this is of limited physiological relevance as it is not activated by thrombin, the principal PAR-1 agonist *in vivo*.

7.6 FUTURE DIRECTIONS

In this series of studies, the role of thrombin receptor activation *in vivo* in the human vasculature has been explored and established for the first time, including the role of the endothelium and comparison of the effect in smokers and non-smokers. Protease-activated receptors appear to be central to a diverse array of physiological and pathophysiological processes including atherothrombotic diseases, cancer metastasis, neuropathic pain and sepsis, with the number of publications on the subjects reflecting this. We will discuss here some of the main areas of research into protease-activated receptors that are of ongoing interest to our group.

7.6.1 RELEVANCE OF PARS TO PATHOPHYSIOLOGY AND CARDIOVASCULAR DISEASES

There is a marked difference in the response to PAR-1 activation in smokers and non-smokers that is likely to be relevant to smokers' increased risk of acute

thrombosis. Studies of PAR expression in patients with inflammatory and atherothrombotic conditions will provide useful information as PAR expression is up-regulated by disease processes such as inflammation [Schouten *et al*, 2008] and atherosclerosis [Nelken *et al*, 1992] and this in turn could cause different responses. It is of great interest to assess the vasomotor and fibrinolytic response to PAR-1 and possibly PAR-2 activation in subjects with other cardiovascular risk factors, such as hyperlipidaemia, diabetes mellitus and in patients with coronary artery disease. Studies in patient groups such as diabetics and patients with coronary artery disease will however be challenging. These groups may have increased baseline levels of platelet activation and impaired vasodilatory and endogenous fibrinolytic responses to PAR-1 activation. Whilst stimulating PAR-1 receptors in healthy volunteers is unlikely to have clinical consequences, the concern would be that in patients with marked atherosclerosis this could potentially have prothrombotic effects.

The role of PAR-3 and PAR-4 in the vasculature has not been established. The lack of a functional PAR-3 agonist and the high concentrations of agonist required to stimulate PAR-4 activation have prevented the *in vivo* study of these receptors, and although their role in man appears fairly limited, further understanding of this would be of interest, in particular clarifying the role of PAR-4.

7.6.2 Relevance of PARs to treatment

The emerging new PAR-1 antagonists/thrombin receptor antagonists will be of great interest, not only to assess antiplatelet effects but also their effects on vasomotor and endogenous fibrinolytic function, both in the presence and absence of PAR-1

stimulation, both in healthy volunteers and patients. Although large phase III clinical trials are conducted to assess antiplatelet effects of thrombin receptor antagonists, and safety and clinical endpoints (see section 7.7), there remains a role for studies looking specifically into vasomotor and fibrinolytic function and the mechanism of these effects.

Direct thrombin receptors are new oral anticoagulant agents that are currently being compared with warfarin, the mainstay of oral anticoagulation for over 60 years. These drugs, unlike thrombin receptor antagonists, inhibit the enzymatic activity of thrombin in the coagulation cascade. Development of the direct thrombin inhibitor ximelagatran was terminated and the drug withdrawn after large clinical trials, mainly due to concerns regarding adverse affects on liver function [Agnelli *et al*, 2009]. More recently the RE-LY trial compared another direct thrombin inhibitor, dabigatran, with warfarin treatment in patients with atrial fibrillation with promising results [Connolly *et al*, 2009]. Both drugs do, however, appear to have in common a very small rise in myocardial infarction. This could be explained by up-regulation of PAR-1 or impaired t-PA release and we are interested in assessing whether platelet and endothelial PAR-1 expression is up-regulated during treatment with direct thrombin inhibitors and thrombin receptor antagonists with associated risk of rebound atherothrombotic events.

7.7 CLINICAL RELEVANCE

The findings described in this thesis have potential implications for the application of novel PAR-1 receptor antagonists that are currently under clinical development as potential antiplatelet therapies. Following successful phase II clinical trials, recruitment is currently taking place for two large phase III clinical trials assessing the effects of the thrombin receptor antagonist (PAR-1 antagonist) TRA-SCH 530348, which has been granted fast track status by the American Food and Drug Administration. Both are large multicentre randomised placebo controlled trials (clinicaltrials.gov). The TRA 2°P-TIMI 50 study will assess approximately 19 500 patients with prior myocardial infarction, stroke or existing peripheral arterial disease. Patients are followed up for at least one year and the endpoints are cardiovascular death, myocardial infarction, urgent coronary revascularisation or stroke. This trial is due to be completed in 2010. The Thrombin Receptor Antagonist Clinical Event Reduction in acute coronary syndrome (TRACER) trial will follow 10,000 patients with non ST-segment elevation acute coronary syndrome taking standard antiplatelet treatment, with or without SCH 530348, over a year and assess the impact on cardiovascular mortality, myocardial infarction, re-hospitalisation for acute coronary syndrome, urgent coronary revascularisation and stroke. This trial is due to be completed in 2011. The findings of these two trials will be very interesting, in particular due to the long follow up period [Angiolillo and Guzman, 2008].

The role of PARs in angiogenesis is an exciting field and remains to be explored further. Protease-activated receptor-1 expression is up-regulated and appears to

correlate directly to the metastatic potential of various cancers including ovarian [Agarwal *et al*, 2008] and breast cancer [Yang *et al*, 2009] and malignant melanoma [Melnikova *et al*, 2009]. This is thought to be related to inflammatory pathways, platelet activation and breakdown of cell-cell barriers, and PAR-1 inhibition may in particular be advantageous in advanced metastatic cancer. Related to its role in angiogenesis PAR-1 inhibition could, however, adversely affect wound healing and tissue ischaemia.

7.8 CONCLUSIONS

In this series of pre-clinical and clinical studies the *in vivo* vascular effects of PAR-1 activation in man have been established for the first time. Activation of PAR-1 has been demonstrated to cause venous constriction, arterial dilatation, platelet activation and release of endogenous t-PA. These intriguing and contrasting effects of PAR-1 agonism reveal the diverse nature of the vascular consequences of thrombin activation in man. The vascular effects of PAR-1 agonism *in vivo* can be understood in terms of limiting intravascular thrombosis and maintaining vessel patency and haemostatic balance: in the presence of a developing intravascular thrombosis, in veins constriction would limit thrombus propagation and embolisation, whereas in the arteries, increased blood flow and endogenous fibrinolysis would limit arterial thrombosis by ensuring thrombus clearance and dissolution. This delicate balance is affected in smokers who have a reduced vasodilatory response to PAR-1 activation and an all but abolished fibrinolytic response, which would enhance clot expansion and vessel occlusion. This could in part explain the increased risk in smokers of

acute thrombotic events. These findings are likely to be directly relevant to the development of thrombin receptor antagonists and their use in patients with ischaemic heart disease.

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7

Direct thrombin inhibitors

INGIBJÖRG J GUDMUNDSDÓTTIR, DAVID E NEWBY

Introduction

Blood coagulation prevents blood loss and has been crucial to survival throughout evolution. It is initiated by 'intrinsic' factors in the blood coming into contact with a foreign surface, such as collagen or subendothelial matrix, or in response to 'extrinsic' factors released by injured tissues, such as tissue factor (Fig. 7.1). These intrinsic and extrinsic pathways converge and lead to thrombin activation. Thrombin is the central enzyme catalysing the conversion of fibrinogen to fibrin, and generates the characteristic fibrin mesh of thrombus.

Although blood coagulation prevents inappropriate haemorrhage and exsanguination following tissue trauma or injury, intravascular thrombosis is a major cause of

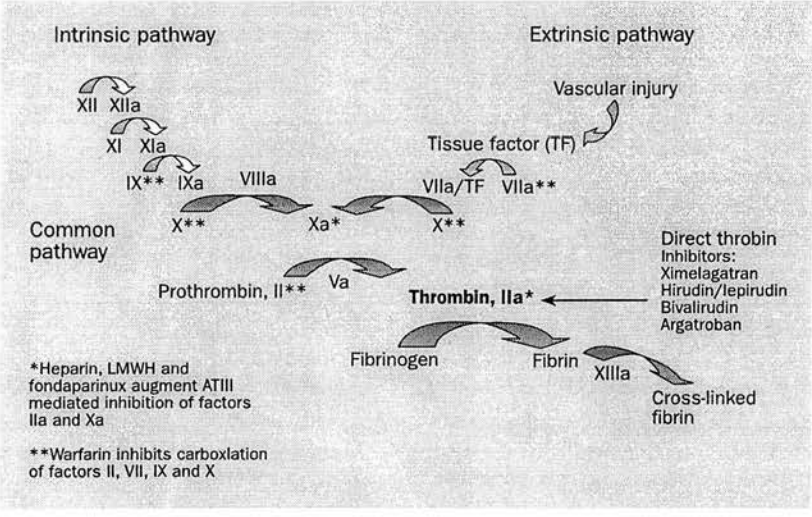

Fig. 7.1 Schematic view of the coagulation cascade.

morbidity and mortality. Arterial thrombosis causes tissue ischaemia including myocardial and cerebral infarction, and is the leading cause of death in Western societies. Venous thrombosis can cause localized tissue oedema but is most serious when it embolises to the lung where it may cause life-threatening pulmonary thromboembolism.

Anticoagulant therapy

Anticoagulant therapy is used in the treatment and prevention of arterial and venous thrombosis in patients with a range of conditions including atrial fibrillation, mechanical prosthetic heart valves, deep venous thrombosis, acute coronary syndromes and pulmonary embolism. Current parenteral and oral anticoagulant therapies tend to act at multiple sites in the coagulation cascade (Table 7.1; Fig. 7.1) and have significant drawbacks that limit their use. Warfarin and heparin are the most commonly used anticoagulant agents but have unpredictable pharmacokinetic and pharmacodynamic actions, potentially serious side effects, and important drug and food interactions. They require regular repeated monitoring and dose adjustments. There is therefore a need for more convenient, predictable and reliable anticoagulant therapies.

This chapter will describe recent data regarding a new class of anticoagulant therapy that acts solely through direct enzymatic inhibition of thrombin: the direct thrombin inhibitors. Although other agents exist (Table 7.1), we will principally consider two new agents: ximelagatran and bivalirudin. Ximelagatran, the pro-drug of melagatran, is the first orally available anticoagulant since the introduction of warfarin over 60 years ago |**1**|. Bivalirudin is a parenteral peptidic agent that is showing particular promise in use during percutaneous coronary intervention (PCI).

Drugs that act earlier in the coagulation cascade, such as the pentasaccharide fondaparinux, are also under development and are now entering clinical use |**2**|. These drugs act indirectly to decrease thrombin generation by factor Xa inhibition and will not be discussed further here.

Intravascular thrombosis and anticoagulation

Anticoagulation is widely used for the treatment and prophylaxis of thrombosis and thromboembolism. Vessel occlusion can occur either in the arterial or venous circulation and the clinical consequences differ depending on the location.

Venous thrombosis

Venous blood flow is slow and under low pressure. Thrombus formation is most common in the calf veins and mainly consists of red blood cells in a mesh of fibrin; so-called 'red thrombus'. Venous thrombosis is therefore readily prevented and treated by inhibiting thrombin-induced fibrin formation.

Consistent with Virchow's triad, any condition that causes venous congestion (reduced flow), trauma (vessel wall damage) or activation of the coagulation system

(hypercoagulability) will predispose to venous thrombosis. There are several well recognized risk factors including major surgery, malignancy (especially adenocarcinoma), immobility, genetic traits and oral contraceptive or hormone replacement therapy |**3**|.

Most venous thrombi of the calves go unnoticed and small emboli are frequently filtered from the circulation in the lungs without causing symptoms. The risk of complications increases steeply if the thrombus extends more proximally and detachment may lead to symptomatic pulmonary embolism.

Venous thromboembolism (VTE) prophylaxis, normally with low doses of heparin, is indicated in immobilized and post-operative patients. Longer treatment is indicated in those with confirmed proximal (above-knee) deep vein thrombosis and pulmonary embolism but the length of treatment varies between 3 months and indefinitely |**4,5**|.

Arterial thrombosis

Thrombus formation in the arterial circulation is often more rapid and even a small thrombus can have devastating effects. Arterial thrombus tends to be caused by mechanical injury or plaque rupture of atherosclerotic arteries. The thrombus is rich in platelets connected by fibrin strands; so-called 'white thrombus'. Agents that minimize either platelet activation or fibrin deposition are very important in the prevention and treatment of arterial thrombus.

Arterial occlusion can be caused by thrombosis *in situ*, as occurs with an acute coronary syndrome, or as a result of thromboembolism, such as a stroke from aortic or cardiac thrombus. In atrial fibrillation, cardiac thrombus forms because of atrial blood stagnation and is not as platelet rich |**6**|. This may, in part, explain why aspirin is less effective in preventing stroke than warfarin in patients with atrial fibrillation |**7**|.

Current treatment

Warfarin

Warfarin blocks the hepatic γ-carboxylation of the vitamin K-dependent clotting factors II, VII, IX and X. The onset of anticoagulant activity is delayed in accordance with the half-life of these factors, 2–3 days. Long-term anticoagulation with warfarin is considered in patients with atrial fibrillation, prosthetic heart valves, rheumatic heart disease, deep vein thrombosis or pulmonary embolism. Patients with atrial fibrillation should be considered for anticoagulation if they have one or more of the following risk factors: previous history of stroke; transient ischaemic attack or systemic embolism; hypertension; diabetes mellitus; impaired left ventricular function; and age over 65 years |**5,8**|. In such patients, warfarin reduces the risk of thromboembolism by 60–70% |**9,10**|.

Treatment with warfarin is complicated by the need to assess liver function at onset and the continuous monitoring of anticoagulant control using the international

Table 7.1 Anticoagulants and their mode of action

	Mode of action	Half-life	Clearance	Side effects non-haemorrhagic	Indications
Unfractionated heparin (UFH)	Binds to and enhances the inhibitory action of antithrombin III (ATIII) against FXa and thrombin (FIIa) Inhibits free but not clot bound thrombin	I.v. inj.: 1/2–1 h S.c. inj: 10–12 h	Renal and hepatic Anticoagulation can be reversed with protamine sulphate	Unpredictable anticoagulation – must be monitored (APTT), heparin induced thrombocytopenia (HIT) in 2–3%, osteoporosis, heparin resistance, skin necrosis, hypersensitivity, rarely hyperkalaemia	Venous thromboembolism (VTE) prophylaxis and treatment acute coronary syndrome (ACS) percutaneous coronary intervention (PCI) After thrombolysis Acute peripheral arterial occlusion Prevention of clotting in extracorporeal circuits
Low molecular weight heparin (LMWH)	Derived from UFH but smaller pentasaccharides and stronger activity against FXa than FIIa. Inhibits free but not clot bound thrombin	Differs between compounds but sufficient for once or twice daily s.c. administration	Renal No antidote available	See heparin Low risk of HIT and osteoporosis Monitoring of coagulation usually not required	VTE prophylaxis and treatment ACS Prevention of clotting in extracorporeal circuits
Warfarin (4-hydroxy-coumarin)	Vitamin K antagonist which inhibits the γ-carboxylation of factors II, VII, XI and X in the liver	20–60 hours	Hepatic and renal Vitamin K slowly reverses anticoagulation. Prothrombin complex concentrate is required in major bleeding (factors II, VII, IX and X)	Delayed onset of action. Drug and food interactions. Binds to plasma proteins. Contraindicated in pregnancy. Hypersensitivity, rash and skin necrosis	Atrial fibrillation (AF), rheumatic heart disease, prosthetic heart valves. Prophylaxis and treatment of VTE

Ximelagatran/ melagatran	Direct thrombin inhibitor. Inhibits free and clot bound thrombin	Oral intake: 4–5 hours	Renal	Elevated liver enzymes in ~6% Monitoring of coagulation not required	VTE prophylaxis and treatment Also proposed for: AF Post MI
Lepirudin (recombinant hirudin)	Direct thrombin inhibitor. Inhibits free and clot bound thrombin.	I.v. inj: 40 min S.c. inj: 120 min	Renal Thrombin inhibition is almost irreversible and no antidote available	Hypersensitivity reaction Monitoring of coagulation required	HIT type II
Bivalirudin	Direct thrombin inhibitor. Inhibits free and clot bound thrombin	I.v. inj: 25 min	Mainly proteolysis but also hepatic and renal	Hypersensitivity reaction Monitoring of coagulation required	During PCI
Argatroban	Direct thrombin inhibitor. Inhibits free and clot bound thrombin	I.v. inj: 45 min	Hepatic	Hypersensitivity reaction Monitoring of coagulation required	HIT type II
Fondaparinux	Synthetic pentasaccharide that binds to and enhances AT activity against FXa. No direct thrombin activity.	S.c. inj: 13–15 hours	Renal No specific antidote available and long half-life.	Abnormal liver function tests. Low risk of thrombocytopenia.	Proposed for prophylaxis and initial treatment of VTE and in ACS.

Source: compiled by present authors.

normalized ratio (INR) of the prothrombin time. Achieving the appropriate level of anticoagulation is hindered by numerous and important drug–drug and drug–food interactions. Warfarin has a narrow therapeutic window and the risk of bleeding increases steeply with a small rise in INR. The risk of major haemorrhage resulting in death, hospitalization, intracranial haemorrhage or the need for blood transfusion with warfarin is about 1–4% per year |**11**|. Warfarin also inhibits production of the anticoagulant factors C and S. If their concentration falls dramatically, such as with excessive loading doses or in people with hereditary Protein C or S deficiency, there is a temporary procoagulant state with the risk of small vessel thrombosis and skin necrosis. Warfarin can also be teratogenic and should be avoided in pregnancy.

Heparin

Heparin inhibits factors Xa and IIa (thrombin) by enhancing the activity of antithrombin III. Unfractionated heparin has largely been replaced by low molecular weight heparin (LMWH) for some indications, such as unstable angina and deep vein thrombosis, but remains the most common anticoagulant in other settings, such as during PCI |**12**|.

There are a number of limitations to the use of heparin. Plasma proteins can bind to heparin and reduce its activity. Anticoagulation with unfractionated heparin is often unpredictable and requires frequent monitoring and dose titration. Antithrombin III only works against free factor Xa and there is little activity against clot-bound factor Xa. Heparin also has a number of important side effects, especially following longer term use, for example osteoporosis and heparin resistance. Heparin can cause platelet activation and one of the most serious non-haemorrhagic side effects is heparin-induced thrombocytopenia (HIT). The diagnosis of HIT should be suspected if there is a >50% unexplained fall in platelet count, if skin lesions form at injection sites, or if the patient develops thrombosis while on heparin. In the common non-immune (type I) form of HIT, the platelet count rarely falls below 100×10^9/l and the patient normally recovers spontaneously. The more serious autoimmune form (type II) of HIT occurs in about 3% of patients who receive heparin for over 5 days |**13**|. In spite of the low platelet count, it is a very prothrombotic state and frequently leads to arterial or venous thrombosis, amputations or death. HIT is much less common with LWMH |**13**| and very unlikely with the new pentasaccharides |**14**|, such as fondaparinux.

Basic science underlying direct thrombin inhibitors

The role of thrombin in coagulation

Thrombin (factor IIa) is generated from prothrombin through activation of both the intrinsic and extrinsic pathways of the coagulation cascade. Thrombin plays an essential role in coagulation through its protease activity, leading to the formation of fibrin from fibrinogen. This is further enhanced by positive feedback loops causing

activation of factor V, VIII and X in the intrinsic pathway and factor VII in the extrinsic pathway. Factor XIII is also activated by thrombin and stimulates cross-linking and stabilization of fibrin (Fig. 7.1).

Thrombin is a direct mediator of platelet activation and vascular inflammation. These effects are largely mediated via a novel group of G-protein coupled receptors called proteinase-activated receptors (PARs) |**15**|. Proteolytic cleavage of the receptor leads to unmasking of a short peptide sequence that remains tethered and activates the receptor leading to a cascade of intracellular signals. To date, four different types of PAR have been identified. PAR-1, -3 and -4 are all activated by thrombin, whereas PAR-2 is mainly activated by trypsin. PAR-1 mediates most of the effects of thrombin in the cardiovascular system and is present on endothelial cells and platelets. In healthy human arteries, PAR-1 appears to be expressed almost exclusively in the endothelial layer, whereas in atheromatous arteries there is a more widespread expression, raising the possibility that PAR-1 receptor activation may contribute to atherogenesis and restenosis.

Direct thrombin inhibitors

The direct thrombin inhibitors bind to the substrate-recognition/and or catalytic site of thrombin and, by virtue of their small size, can bind to and inactivate both free and clot-bound thrombin. They are not inactivated by plasma proteins and are not affected by other drugs or food. They therefore have a more predictable anticoagulant effect, which requires less monitoring. Direct thrombin inhibitors also have antiplatelet effects |**16**| and may augment thrombolysis induced by fibrinolytic agents |**17**|. Melagatran and related agents bind to thrombin in an extended conformation at three different sites, whereas argatroban and related drugs bind to thrombin in a more compact way (Fig. 7.2).

Several direct thrombin inhibitors are in development but only a few have been studied in large trials. Ximelagatran, the oral prodrug of melagatran, has been studied most extensively. Other oral agents, such as BIBR 1048 |**18**| and SSR182289A 1 |**19**|, are promising direct thrombin inhibitors that are in the very early stages of clinical development.

Three parenteral direct thrombin inhibitors are currently available: hirudin/lepirudin, bivalirudin and argatroban. Hirudin was originally isolated from salivary glands of the medicinal leech in 1904, but is now available in a recombinant form as lepirudin |**20**|. Further modifications have also been made to generate a hirudin analogue, bivalirudin. Clinical studies in the 1990s assessing the use of hirudin in patients with ischaemic heart disease |**21–24**| showed an increased risk of bleeding when doses were sufficient to have therapeutic benefit. Argatroban is a small synthetic molecule |**25**| that binds reversibly to the catalytic site of thrombin. Caution is required with use in patients with impaired liver function because of its biliary excretion but it is safe to use in patients with renal impairment |**26**|. Hirudin and argatroban are indicated in patients with heparin-induced thrombocytopenia and a recent study suggests that argatroban is a safe anticoagulant in patients with acute ischaemic stroke, although efficacy has not been established |**27**|.

NEW FIG 7.2 TO COME
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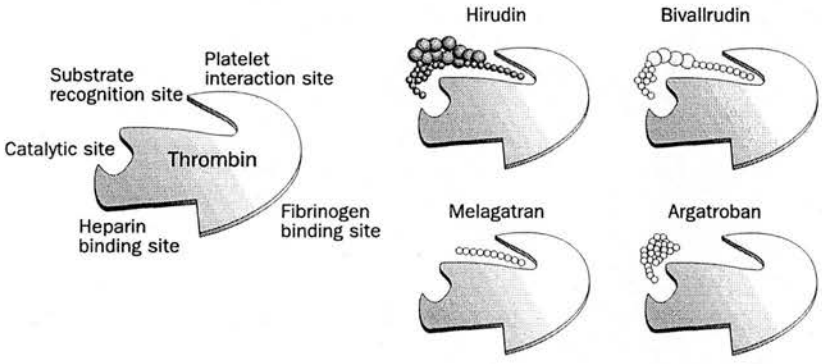


Fig. 7.2 (a) A schematic representation of the modes of inhibition of (a) heparin and (b) direct thrombin inhibitors of fibrin-bound thrombin. (b) Chemical structure of argatroban (to the left) and argatroban and active site region (right) of the complexes formed between thrombin (yellow connections) and argatroban (orange connections). Source: (a) To be modified from Weitz JI, *Thrombosis Research* 2003; **109**: S17–S22. (b) Hauptmann J and Stürzbecher J, *Thrombosis Research* 1999; **93**: 203–41.

Melagatran/ximelagatran

Melagatran is a selective competitive inhibitor of human thrombin and inhibits both free and clot-bound thrombin. Protecting groups have been added to melagatran to form ximelagatran (Fig. 7.3), making it an uncharged and more lipophilic molecule, which has better bioavailability |**28,29**|. Ximelagatran can be crushed or dissolved in water and given via nasogastric tube for patients with swallowing difficulties |**30**|. It is rapidly absorbed and biotransformed to melagatran, reaching peak plasma concentration 1.5–2.0 h after intake. Unlike vitamin K antagonists, where introducing full anticoagulation takes 4–5 days, anticoagulation is almost immediate. Melagatran is not metabolised further and is renally excreted with a half-life of 4–5 h |**31**|. Excretion is delayed in patients with impaired renal function. Melagatran prolongs blood coagulation as measured by various clotting tests such as the activated partial thromboplastin time (APTT) and prothrombin time. There is no interaction with the hepatic cytochrome P_{450} (CYP) system |**32**| and therefore very few drug–drug interactions. No specific antidote is available but anticoagulation is reversed within 1 day of stopping treatment because of its short half-life.

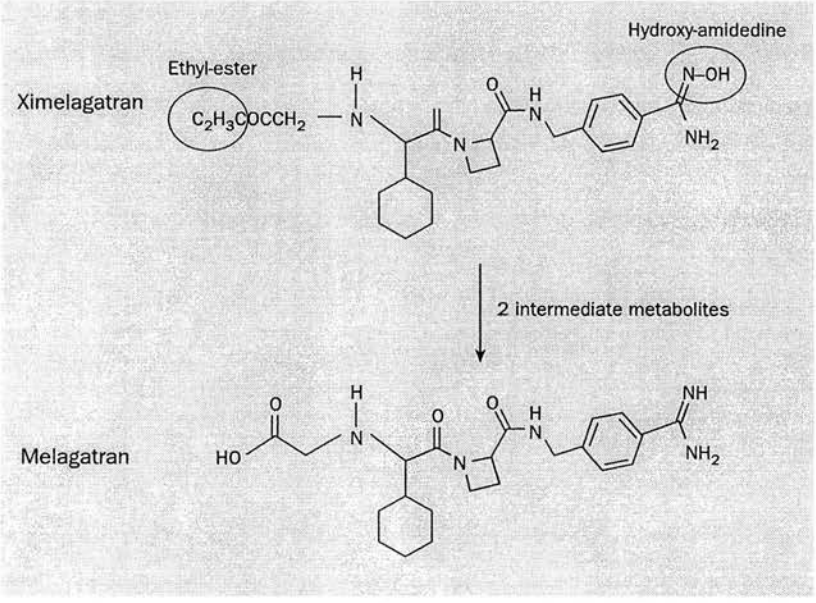


Fig. 7.3 Molecular structure of ximelagatran and melagatran. Source: Samama MM, *et al. Pathophysiol Haemost Thromb* 2002; **32**: 218–24.

About 6–7% of patients in clinical trials have developed derangements in liver function tests (aminotransferase levels greater than threefold the upper limit of normal) and this has been the most frequent side effect. Liver function tests typically become abnormal 2–6 months into treatment and in most instances revert to normal either spontaneously or after treatment cessation. However, there is evidence that ximelagatran can induce severe liver injury and this is a major concern. A recent review from the American Food and Drug Administration (FDA) concluded that the potential risk of side effects, mainly deranged liver function, might outweigh the benefits of ximelagatran and, on this basis, did not support the manufacturer's application for a licence |**33**|. Further work on the side effect profile of this drug will be required. At present, the mechanism and the long-term effects of the hepatic injury are unknown.

Bivalirudin

Bivalirudin is a recombinant polypeptide analogue of the carboxy-terminal of hirudin |**34**|. It is a direct thrombin inhibitor given by intravenous infusion and forms a bivalent complex with thrombin. The connection is slowly reversible as a result of proteolytic cleavage by thrombin |**35**|. Bivalirudin is therefore partly broken down by this catalytic mechanism and is, to a smaller extent, excreted through the kidneys and liver. It has a short half-life of around 25 min |**36**|. Unlike hirudin, the binding of bivalirudin to thrombin is reversible and potentially makes bivalirudin safer to use.

Recent studies assessing efficacy and safety of ximelagatran

Stroke prevention with the oral direct thrombin inhibitor ximelagatran compared with warfarin in patients with non-valvular atrial fibrillation (SPORTIF III): randomised controlled trial

SB and Executive Steering Committee on behalf of the SPORTIF III Investigators. *Lancet* 2003; **362**: 1691–8

BACKGROUND. The aim of the study was to establish whether ximelagatran represented a potential alternative and was non-inferior to warfarin, within a margin of 2% per year for prevention of stroke and systemic embolisation. A total of 3410 patients from 23 countries, with non-valvular atrial fibrillation and one or more risk factors for stroke were studied. Patients were treated in an open-label design with either warfarin or ximelagatran (36 mg twice daily). Concomitant aspirin up to 100 mg daily was allowed but other anticoagulants prohibited. Primary end-points were stroke (ischaemic or haemorrhagic) or systemic embolism. Secondary

Table 7.2 Recent studies assessing efficacy and safety of ximelagatran

Study	Aim	Primary end-point	Absolute risk reduction	Conclusion	Comments
SPORTIF III *n* = 3410	Ximelagatran vs warfarin for stroke prevention in patients with non-valvular AF	Stroke or systemic embolism 2.3% warfarin vs 1.6% ximelagatran	0.7% CI —0.1 to 1.4% *P* = 0.1000	Ximelagatran non-inferior to warfarin	
ESTEEM *n* = 1883	Ximelagatran and aspirin vs aspirin alone for secondary prophylaxis after MI	All-cause mortality, non-fatal myocardial infarction and severe recurrent ischaemia. 16.3% warfarin vs 12.7% ximelagatran	3.6% CI 0.59–0.98 *P* = 0.036	Ximelagatran more efficient than placebo but increased risk of (mainly minor) bleeding	
EXPRESS *n* = 2835	Melagatran/ximelagatran vs enoxaparin for prevention of VTE after total hip or knee replacement	1st stage: major VTE 2.3% warfarin vs 6.3% enoxaparin 2nd stage: All VTE 20.3% warfarin vs 26.6% enoxaparin	1st stage: 4.0%; CI 5.6–2.4%; *P* = 0.0000018 2nd stage: 6.3%: CI 9.7–2.9% *P* <0.0004	Ximelagatran non-inferior/ superior to enoxaparin in preventing major and total VTE	Severe bleeding more common in the ximelagatran group (3.3% vs 1.4%), mainly after THR
METHRO III *n* = 2788	Melagatran/ximelagatran vs enoxaparin for prevention of VTE after total hip or knee replacement	All VTE 31.0% ximelagatran vs 27.3% enoxaparin	Absolute risk reduction 3.7% in favour of enoxaparin *P* = 0.053	Similar event rate but a trend for patients receiving enoxaparin to have fewer VTE	Slightly different dosing regime to EXPRESS
THRIVE I *n* = 350	Ximelagatran vs warfarin and dalteparin for treatment of DVT for the first 14 days after diagnosis. Dose finding study	Change in thrombus size Regression in thrombus size in 69% in both groups	No statistically significant difference	Similar results (symptoms and thrombus size) for all doses of ximelagatran tested and warfarin	THRIVE II and V are ongoing larger studies on patients with DVT/PE
THRIVE III *n* = 1233	Ximelagatran vs placebo for secondary prevention of DVT/PE after conventional anticoagulation for 6 months	Symptomatic recurrent VTE 2.8% ximelagatran, 12.6% placebo	Hazard ratio 0.16; CI 0.09–0.30; *P* <0.001	Lower risk of recurrence in ximelagatran group	Bleeding not significantly increased
EXULT A *n* = 1851	Ximelagatran (24 or 36 mg) vs warfarin for VTE prophylaxis after total knee replacement	VTE and death 20.3% for 36 mg ximelagatran vs 27.6% for warfarin	7.3% CI 12.0–2.5% *P* = 0.003	Lower risk of VTE in ximelagatran group, main reduction was in distal DVTs	Anticoagulation was started 12–24 h after surgery
Colwell *et al.* *n* = 1557	Ximelagatran 24 mg vs enoxaparin for VTE prevention after total hip replacement	All VTE 7.9% ximelagatran 4.6% enoxaparin	3.3% in favour of enoxaparin; CI 0.9–5.7%	The study failed to confirm non-inferiority of ximelagatran	No melagatran was given peri-operatively in this study

Source: compiled by present authors.

end-points were composites of: (1) major and minor bleeding; (2) treatment discontinuation; (3) ischaemic stroke, transient ischaemic attack and systemic embolism; (4) acute myocardial infarction (MI), stroke, systemic embolism and death.

INTERPRETATION. Systemic embolism occurred in 2.3%/year in the warfarin group and 1.6%/year in the ximelagatran group, absolute risk reduction of 0.7% (95% confidence interval [CI] –0.1 to 1.4; $P = 0.1000$). Composite of mortality, stroke, systemic embolism and MI was 4.2% in the ximelagatran group and 4.9% in the warfarin group. The rate of major or minor bleeding was 25.8% in the ximelagatran group and 29.8% in the warfarin group ($P = 0.0065$; absolute risk reduction 4.0% with 95% CI –6.9 to –1.1). Fourteen per cent of participants had to terminate treatment early in the warfarin group and 18% in the ximelagatran group, mainly because of elevation of liver transaminases. Ximelagatran was non-inferior to warfarin for the prevention of thromboembolic events in patients with non-valvular atrial fibrillation.

Comments

Atrial fibrillation is the most common sustained cardiac arrhythmia, occurring in about 6% of the population over 65 years of age |**37**|. Results of the AFFIRM and RACE studies |**38,39**| have emphasized the importance of rate control and anticoagulation in patients with recurrent atrial fibrillation rather than trying to obtain or maintain sinus rhythm. Studies have consistently confirmed that anticoagulation with warfarin is underutilized in spite of clear benefits in patients with atrial fibrillation, and treatment that is easier to manage could improve this.

The major limitations of the SPORTIF III trial are its open-label design and the wide non-inferiority margin (2% absolute difference) proposed by the investigators. Open-label studies are likely to overestimate potential treatment benefits. The SPORTIF V study is a double-blind, randomized, controlled trial that was carried out on 3922 patients in the USA and Canada, and replicated SPORTIF III in a more rigorous manner. The results of SPORTIF V have not yet been formally published. An abstract |**40**| indicates that the results are similar to SPORTIF III, although the stroke and embolic event rate in the warfarin group appeared to be slightly lower than that with ximelagatran (absolute difference 0.45%; 95% CI –0.13 to 1.03). A further ongoing study that is addressing the issue of anticoagulation in atrial fibrillation is the ACTIVE trial, in which warfarin is compared with aspirin and clopidogrel.

Oral ximelagatran for secondary prophylaxis after myocardial infarction: the ESTEEM randomised controlled trial

Wallentin L, Wilcox RG, Weaver WD, *et al.*, for the ESTEEM Investigators. *Lancet* 2003; **362**: 789–97

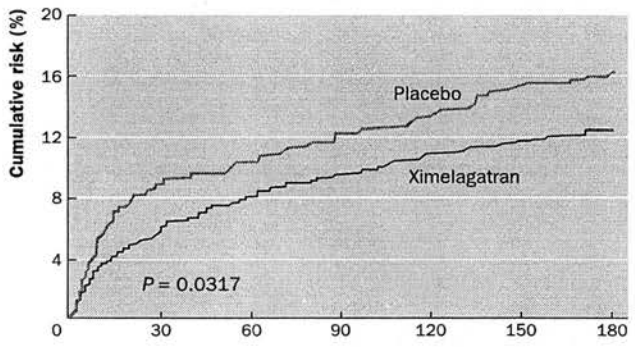
BACKGROUND. The aim was to assess the effectiveness of ximelagatran combined with aspirin against aspirin alone after MI. This was a placebo-controlled, dose-guiding, multicentre study of 1883 patients with ST-elevation or

non-ST-elevation MI within the previous 14 days. Study participants also had at least one thromboembolic risk factor. Among exclusion criteria were PCI in the previous 4 months or planned PCI within 60 days, or if treatment with other antiplatelet or anticoagulant drugs was indicated. Patients were randomized to receive either one of four doses of ximelagatran (24, 36, 48 or 60 mg twice daily) or placebo for 6 months. All patients received 75–160 mg aspirin daily.

INTERPRETATION. Ximelagatran combined with aspirin was more effective than aspirin alone in reducing the risk of the primary end-point of death, non-fatal MI or severe recurrent ischaemia (12.7% for ximelagatran vs 16.3% for placebo; $P = 0.0357$; Fig. 7.4). There was no apparent difference in effect between the individual doses of ximelagatran. A *post hoc* analysis found that ximelagatran reduced the cumulative risk of death, non-fatal MI and non-fatal stroke from 11.1% to 7.4% (hazard ratio [HR] 0.66; 95% CI 0.48–0.90). There was no difference in the risk of major bleeding, 0.9% in placebo group versus 1.8 % in the ximelagatran group. However, combined major and minor bleeding was more common in the ximelagatran group (22% compared with 13% in placebo group; HR 1.76, 95% CI 1.38–2.25). Treatment was stopped in 7% of patients taking ximelagatran because of a rise in liver enzymes, but this was less common in patients taking the lowest dose of 24 mg twice daily than in patients taking higher doses.

Comments

This phase II study compared the combination of aspirin with either ximelagatran or placebo rather than another antithrombotic agent such as warfarin. This limits the value of the study as it has previously been shown that the combination of warfarin



Patients at risk							
Placebo	638	565	549	526	515	501	417
Ximelagatran	1245	1134	1100	1069	1033	1010	820

Fig. 7.4 ESTEEM trial. Cumulative risk of death, myocardial infarction, and severe recurrent ischaemia. All ximelagatran doses pooled. Analysis by intention to treat.
Source: Wallentin L, Wilcox RG, Weaver WD, *et al. Lancet* 2003; **362**: 789–97.

with aspirin is superior to aspirin alone in preventing death and thromboembolic events after acute MI |**41**|. Furthermore, a considerable proportion of patients did not qualify for the study as they were excluded if antithrombotic agents other than aspirin or a PCI were indicated.

A recent report for the FDA |**33**| where adverse events were pooled and analysed (*post hoc*), raised concern that the incidence of coronary events was higher in the patients treated with ximelagatran. These data are mainly drawn from a group of patients receiving prophylactic treatment for VTE after total knee replacement (EXULT A and B studies) for a brief period and the numbers, although statistically significant, are low. The underlying mechanism is also unclear, although one possibility is a rebound prothrombotic effect after drug withdrawal.

The direct thrombin inhibitor melagatran followed by oral ximelagatran compared with enoxaparin for the prevention of venous thromboembolism after total hip or knee replacement: The EXPRESS study

Eriksson BI, Agnelli G, Cohen AT, *et al.*, on behalf of the EXPRESS study group. *J Thromb Haemost* 2003: **1**: 2490–6

Background. A total of 2835 patients undergoing total hip or knee replacement at 77 centres were randomized in a double-blind manner to receive VTE prophylaxis, comparing melagatran followed by ximelagatran to enoxaparin. Melagatran, 2 mg, was given subcutaneously immediately prior to surgery, 3 mg at least 8 h later and then oral ximelagatran 24 mg twice daily. Patients receiving enoxaparin were given the first dose of 40 mg subcutaneously 12 h before surgery and then once daily. Prophylaxis was continued for 8–11 days when a venogram was performed. Other anticoagulant or antiplatelet agents were prohibited apart from non-steroidal anti-inflammatory drugs including aspirin. The first-stage primary efficacy outcome (to show non-inferiority within 2%) was major VTE during the treatment period: proximal deep venous thrombosis, pulmonary embolism and/or death where pulmonary embolism could not be ruled out. The second-stage primary efficacy outcome (designed to show superiority of melagatran/ximelagatran) was: all deep venous thrombosis (proximal or distal), pulmonary embolism and/or death from any cause.

Interpretation. About 80% of patients had venograms that could be appropriately analysed. The rate of major deep venous thrombosis was lower in patients given ximelagatran, 2.3% versus 6.3% in the enoxaparin group (absolute risk reduction 4.0%; $P = 0.0000018$), although there was a smaller benefit in favour of ximelagatran in reducing rate of total deep venous thrombosis (20.3% vs 26.6%; $P < 0.0004$). The rate of symptomatic deep venous thrombosis and pulmonary embolism was lower than 1% in both groups during the study period. More patients in the ximelagatran (3.3%; 95% CI 2.4–4.4) than enoxaparin group (1.4%; 95% CI 0.7–1.9) had severe bleeding. The investigators' conclusion was that melagatran/ximelagatran was non-inferior and was indeed superior to enoxaparin for VTE prophylaxis.

Comments

Increased bleeding was seen mainly in patients undergoing total hip replacement and not in patients having total knee replacement. This might be explained by the fact that an arterial tourniquet is commonly applied during knee replacement surgery. This additional bleeding risk did not cause increased mortality or wound complications.

Large orthopaedic operations such as total hip and knee replacements carry a high risk of post-operative VTE. Current guidelines recommend at least 7–10 days of prophylactic anticoagulation but there is evidence that VTE prophylaxis for 4–5 weeks after the operation is beneficial |**42,43**|. More convenient anticoagulants might increase the use of prolonged prophylactic treatment.

Direct thrombin inhibitor melagatran followed by oral ximelagatran in comparison with enoxaparin for prevention of venous thromboembolism after total hip or knee replacement: The METHRO III study

Eriksson BI, Agnelli G, Cohen AT, *et al. J Thromb Haemost* 2003; **89**: 288–96

BACKGROUND. The study compared melagatran/ximelagatran with enoxaparin for VTE prophylaxis after total hip or knee replacement. Two thousand seven hundred and eighty-eight patients were randomized to receive for 8–11 days: either 3 mg melagatran s.c. starting 4–12 h post-operatively, followed by oral ximelagatran 24 mg twice daily; or enoxaparin 40 mg s.c. once daily. The primary efficacy end-point was VTE (composite of deep-vein thrombosis detected by mandatory venography, pulmonary embolism or unexplained death).

INTERPRETATION. Venous thromboembolism occurred in 31.0% in the ximelagatran group compared with 27.3% in the enoxaparin group, an absolute risk reduction of 3.7% in favour of enoxaparin ($P = 0.053$). The incidence of bleeding was similar.

Comments

It was on the basis of this study that a slightly different dosing regime was used in the EXPRESS study. In the METHRO III study the melagatran was started 4–12 h post-operatively, but was started immediately prior to surgery in EXPRESS. This might explain the observation that patients receiving ximelagatran in the EXPRESS study had a slightly higher risk of bleeding but lower risk of VTE than in the METHRO III study. Studies on new anticoagulant agents are often first carried out in patients undergoing large orthopaedic operations, as the risk of venographically proven VTE is high and fewer participants are thus required to reach a statistically significant difference.

A randomized, controlled, dose-guiding study of the oral direct thrombin inhibitor ximelagatran compared with standard therapy for the treatment of acute deep vein thrombosis: THRIVE I

Eriksson H, Wåhlander K, Gustafsson D, Welin LT, Frison L, Schulman S. *J Thromb Haemost* 2003; **1**: 41–7

BACKGROUND. This was a phase II dose-finding study of 350 individuals with acute deep venous thrombosis comparing ximelagatran (24, 36, 48 or 60 mg twice daily) with LWMH followed by warfarin. Treatment was started at diagnosis and continued for 14 days, when a second venogram was performed.

INTERPRETATION. Similar changes in thrombus size and clinical symptoms occurred in all groups. No difference in bleeding was detected and major bleeding was rare.

Comments

THRIVE II and V are currently ongoing double-blind phase III studies for treatment of deep vein thrombosis and/or pulmonary embolism that may give further evidence for the use of ximelagatran in this large group of patients. They are also likely to provide valuable data regarding potential adverse effects including MI.

Secondary prevention of venous thromboembolism with the oral direct thrombin inhibitor ximelagatran. THRIVE III

Schulman S, Wåhlander K, Lundström T, Billing Clason S, Erikson H for the THRIVE III Investigators. *N Engl J Med* 2003; **349**: 1713–21

BACKGROUND. Ximelagatran was compared with placebo for secondary prophylaxis of deep vein thrombosis. One thousand two hundred and thirty-three patients who had already received conventional anticoagulation for 6 months after a confirmed deep vein thrombosis or pulmonary embolus were randomized to receive either ximelagatran 24 mg twice daily or placebo for 18 months. Objective tests were undertaken if there was a clinical suspicion of new deep venous thrombosis or pulmonary embolism. People with cancer were included in the study unless they had a clear indication for continuing anticoagulation.

INTERPRETATION. Ximelagatran significantly reduced the rate of recurrent thromboembolic events compared with placebo (2.8% vs 12.6%; HR 0.16, 95% CI 0.09–0.30; $P < 0.001$) and this risk reduction continued to increase over time. Rate of bleeding was low (<1%) and similar in both groups.

Comments

There is an increased risk of recurrence after a first deep vein thrombosis but prolonged treatment has traditionally not been given unless major risk factors are present. Previous studies comparing placebo with long-term warfarin have also found reduced incidence of recurrent thromboembolism [**44–46**]. Direct comparison is however difficult as the duration of treatment differs. A trial designed to compare warfarin and ximelagatran would be appropriate.

Comparison of ximelagatran with warfarin for the prevention of venous thromboembolism after total knee replacement

Francis CW, Berkowitz SD, Comp PC, *et al.* for the EXULT A Study Group. *N Engl J Med* 2003; **349**: 1703–12

BACKGROUND. This study followed an initial phase III trial which compared ximelagatran 24 mg twice daily to warfarin after total knee replacement and found ximelagatran to be at least as effective. The aim of this study was to determine whether a higher dose of ximelagatran would be more effective than warfarin without compromising safety. In this double-blind, multicentre study, 1851 patients were randomized to receive either ximelagatran 24 mg twice daily, ximelagatran 36 mg twice daily or warfarin for 7–12 days after total knee replacement.

INTERPRETATION. Ximelagatran 36 mg twice daily was more effective than warfarin in preventing the primary composite end-point of VTE and death from all causes (20.3 % vs 27.6%; $P = 0.003$). There was no difference for the composite secondary end-point of proximal deep vein thrombosis, pulmonary embolism and death or the rate of major bleeding.

Comments

The primary end-point benefit was principally driven by a reduction in the rates of distal deep vein thromboses, as diagnosed by venography. Such venous thromboses are unlikely to cause pulmonary embolism or death. Ximelagatran was here compared with warfarin rather than LMWH for VTE prophylaxis after total knee replacement. This study is therefore mainly of relevance in the USA where warfarin is commonly used as an anticoagulant after orthopaedic operations, whereas LMWH is favoured in Europe. Furthermore, it takes warfarin 3–5 days to reach therapeutic levels and INR levels were below the target range (1.8–3.0) in 35% of patients by day three and 24% at the time of venography, highlighting how frequently patients receiving warfarin are undertreated.

Comparison of ximelagatran, an oral direct thrombin inhibitor, with enoxaparin for the prevention of venous thromboembolism following total hip replacement. A randomized, double-blind study

Colwell CW, Berkowitz SD, Davidson BL, *et al. J Thromb Haemost* 2003; **1**: 2119–30

Background. The aim of the study was to compare the efficacy and safety of ximelagatran to enoxaparin following total hip replacement, with non-inferiority pre-specified at 5%. This was a double-blind trial from 126 centres, mainly in the USA and Canada. Starting from the morning after total hip replacement, 1838 patients were randomized to either oral ximelagatran 24 mg twice daily or enoxaparin 30 mg s.c. twice daily for 7–12 days. Venography was performed on day 12. The primary end-point was all venous thromboembolic events (deep vein thromboses that were symptomatic or detected by routine venography and all pulmonary emboli). Secondary end-points were proximal deep vein thromboses and/or pulmonary emboli as well as bleeding events.

Interpretation. The incidence of VTE was higher in the ximelagatran group (7.9% versus 4.6%; absolute difference 3.3% in favour of enoxaparin; 95% CI 0.9–5.7). Proximal deep vein thromboses and/or pulmonary emboli occurred in 3.6% in the ximelagatran group vs 1.2% in the enoxaparin group (absolute difference 2.4%; 95% CI 0.9–3.9). No significant difference of bleeding was found. Elevated liver enzymes were more common in the group that received enoxaparin.

Comments

The trial failed to show non-inferiority (within 5%) of ximelagatran at a dose of 24 mg twice daily versus enoxaparin in preventing VTE. The absolute difference was 3.3% in favour of enoxaparin with the upper limit of the 95% CI at 5.7%. Ximelagatran does not appear to be as effective as LMWH when administered post-operatively but it should be emphasized that patients in this study did not receive peri-operative melagatran, which is in contrast to the EXPRESS study. Oral factor Xa inhibitors are also in development; comparison of these with LWMH will be of particular interest.

Recent studies assessing efficacy and safety of parenteral direct thrombin inhibitors (Table 7.3)

Table 7.3 Recent studies assessing efficacy and safety of parenteral direct thrombin inhibitors

Study	Aim	Primary end-point	Absolute risk reduction	Conclusion	Comments
REPLACE-1 $n = 1056$	Bivalirudin vs heparin during urgent or elective PCI. Optional GPIIb/IIIa blockade in both groups (72%). Pilot study	Death, MI, repeat revascularization before discharge or within 48h. 5.6% bivalirudin 6.9% heparin	No significant difference $P = 0.40$	Bivalirudin non-inferior to heparin during PCI	
REPLACE-2 $n = 6010$	Bivalirudin and provisional (7.2%) GPIIa/IIIb blockade vs heparin and planned (95%) GPIIa/IIIb blockade during elective or urgent PCI	30 day incidence of death, MI, urgent repeat revascularization, or in-hospital major bleeding. 9.2% bivalirudin 10% heparin + GPIIa/IIIa inhibitor	Odds ratio 0.92; CI 0.77–1.09; $P = 0.32$	Bivalirudin alone non-inferior to heparin + GPIIa/IIIb blocker	Patients with acute MI or unstable angina were not included in the study
Lewis *et al.* $n = 418$	Argatroban vs historical control (mainly heparin cessation) for treatment of heparin-induced thrombocytopenia (HIT)	Death, amputation or new thrombosis. HIT 28.0 vs control 38.8% HIT with thrombosis (HITTS) 41.5% vs control 56.5%	HIT: $P = 0.04$ HITTS: $P = 0.07$	Argatroban reduced incidence of new thrombosis	
ARGIS-1 $n = 171$	Safety of two doses of argatroban vs placebo in patients with acute ischaemic stroke	Symptomatic intracranial haemorrhage (ICH) at 30 days Argatroban: high dose 5.1%, low dose 3.4%, placebo 0%	$P > 0.18$	Difference was not statistically significant	No significant difference between asymptomatic ICH, major haemorrhage or 90-day mortality

Source: compiled by present authors.

Comparison of bivalirudin versus heparin during percutaneous coronary intervention (the randomized evaluation of PCI linking angiomax to reduced clinical events [REPLACE] -1 trial)

Lincoff MA, Bittl JA, Kleiman NS, *et al*. *Am J Cardiol* 2004; **93**: 1092–6

Background. This was a pilot study carried out prior to REPLACE-2. One thousand and fifty-six patients in the USA undergoing PCI were randomized to heparin or bivalirudin during the procedure. All patients received aspirin. Clopidogrel was encouraged and received by 56% before and 90% after the procedure. Glycoprotein (GP) IIa/IIIb inhibitors were planned but at the physicians' discretion, and were given in 72% of cases. Use of clopidogrel and GPIIa/IIIb inhibitors was very similar between the groups.

Interpretation. Composite end-point of death, MI and repeat revascularization before hospital discharge or within 48 h occurred in 5.6% of the bivalirudin versus 6.9% in the heparin group ($P = 0.40$). Bleeding rate was similar but activated clotting times were higher in the group that received bivalirudin (359 vs 293 sec; $P < 0.001$), independent of GPIIa/IIIb inhibitor use.

Comments

This was a pilot study and treatment was not blinded. A statistically significant difference was not established between the two groups. Subgroup analyses indicated that bleeding rates were reduced with bivalirudin compared with heparin in those patients who did not receive concomitant GPIIa/IIIb inhibitors, whereas there was a tendency to reduce ischaemic events regardless of GPIIa/IIIa inhibitor use. GPIIa/IIIb inhibitors were therefore used on a provisional rather than planned basis in REPLACE-II.

Bivalirudin and provisional glycoprotein IIb/IIIa blockade compared with heparin and planned glycoprotein IIb/IIIa blockade during percutaneous coronary intervention. REPLACE-2 randomized trial

Lincoff MA, Bittl JA, Harrington RA, *et al*. *JAMA* 2003; **289**: 853–63

Background. The aim of the study was to compare the direct thrombin inhibitor bivalirudin and provisional GPIIb/IIIa inhibitor with heparin and planned GPIIb/IIIa inhibitor during urgent or elective PCI. All patients received aspirin and clopidogrel for at least 30 days after the procedure and a loading dose before PCI was also encouraged. The primary composite end-point was 30-day incidence of death, MI, urgent repeat revascularization or in-hospital major bleeding. The secondary composite end-point was death, MI or urgent repeat revascularization within 30 days.

Interpretation. In a double-blind manner, 3008 patients were randomized to heparin plus GPIIb/IIIa inhibitor and 2994 patients to bivalirudin. In the bivalirudin group, only 7.2% were given a GPIIb/IIIa inhibitor. Patients in the bivalirudin group had higher activated clotting time values. Death, MI, urgent repeat vascularization or in-hospital major bleeding occurred in 10% in the heparin plus GPIIb/IIIa group and in 9.2% of the bivalirudin group (P = NS). Secondary end-points were also similar, 7.1% for heparin plus GPIIb/IIIa compared with 7.6% for bivalirudin. Thrombocytopenia (platelet count <100) was more common in the heparin plus GPIIb/IIIa group (1.7 vs 0.7%; P <0.001).

Comments

The main strength and aim of this study was to compare bivalirudin to heparin using aggressive antiplatelet strategy with aspirin, clopidogrel and GPIIa/IIIb inhibitors.

There was a small numerical (0.5%) but non-significant difference in ischaemic events. Bleeding was less common in the bivalirudin group, mostly as a result of reduced bleeding at the site of vascular access (2.5% vs 0.8%; P <0.001) as well as minor bleeding (25.7% vs 13.4%; P <0.001). Patients with acute MI or unstable ischaemia were excluded from the trial and these results should not be extrapolated to this clinical setting. Only a small minority of patients in the bivalirudin group (7.2%) received a GPIIb/IIIa inhibitor, and bivalirudin alone would be less expensive than combined heparin plus GPIIb/IIIa inhibition. Bivalirudin, unlike heparin, is likely to reduce thrombin-mediated platelet activation, which would be beneficial during PCI.

It is of particular interest that 85% of patients received pre-treatment with a thienopyridine, predominantly clopidogrel. The recent ISAR-REACT study |**47**| of elective PCI showed that there was no additional benefit of giving a GPIIb/IIIa inhibitor in patients pre-treated with clopidogrel. The ISAR-REACT trial therefore raises the question of whether the results of REPLACE-2 reflect the beneficial effects of thienopyridine use rather than the equivalence of bivalirudin and a GPIIb/IIIa inhibitor. However, a recent re-analysis of the REPLACE-2 data suggests that the benefits of bivalirudin over heparin plus GPIIb/IIIa blockade are not influenced by clopidogrel pre-treatment |**48**|.

Argatroban anticoagulation in patients with heparin-induced thrombocytopenia

Lewis BE, Wallis DE, Leya F, Hursting MJ, Kelton JG for the Argatroban-915 Investigators. *Arch Intern Med* 2003; **163**: 1849–56

Background. This was a prospective non-randomized study treating 418 patients with HIT with argatroban for 5–7 days. This was compared with a historical control cohort of 185 people. Patients were divided into two groups: those with isolated HIT (n = 189) or HIT with thrombosis (HITTS; n = 229). Activated partial thromboplastin time was used to monitor anticoagulation. The primary end-point was a composite of death from all causes, amputation or new thrombosis in 37 days.

Interpretation. There was a statistically significant difference in the occurrence of the primary end-point in the isolated HIT study group who received argatroban compared with control (28.0% vs 38.8%; $P = 0.04$) but not in the HITTS group where 41.5% of argatroban-treated patients had a primary composite end-point versus 56.5% of controls ($P = 0.07$). There was, however, a significant reduction of recurrent thrombosis in both the HIT ($P = 0.02$) and HITTS groups ($P = 0.008$), as well as a reduction in death as a result of thrombosis in the HITTS group. The rates of bleeding were similar.

Comments

There are inherent limitations in using a historical control group, in particular when there is little information about how the control group was treated, mainly cessation of heparin. Low molecular weight heparin and warfarin are contraindicated in HIT because of the risk of cross-reaction with heparin antibodies or worsening thrombosis |**49**|. Direct thrombin inhibitors are thus a promising treatment option for this challenging condition and it would be interesting to see comparisons with other direct thrombin inhibitors.

Argatroban anticoagulation in patients with acute ischaemic stroke (ARGIS-1). A randomized, placebo controlled safety study

LaMonte MP, Nash ML, Wang DZ, *et al.* for the ARGIS-1 Investigators. *Stroke* 2004; **35**: 1677–82

Background. The aim of the study was to assess the safety of argatroban anticoagulation in acute ischaemic stroke. This was a multicentre, randomized, double-blind study of 171 patients with acute stroke. Patients received intravenous argatroban at either high or low dose or placebo for 5 days. The primary outcome was symptomatic intracranial haemorrhage at 30 days. Activated partial thromboplastin times were monitored during treatment.

Interpretation. The incidence of symptomatic intracranial haemorrhage was not significantly different (high dose argatroban 5.1%, low dose argatroban 3.4%, placebo 0%; $P > 0.18$) and neither was the incidence of asymptomatic intracranial haemorrhage, major systemic haemorrhage or 90 day mortality.

Comments

These results are in contrast with studies that have shown an increased risk of bleeding complications after heparin or LMWH in patients with ischaemic stroke. It is also concerning that there was a trend of increased intracranial haemorrhage in the argatroban groups, although this was not statistically significant. This was a safety study and did not have the power to assess the efficacy of argatroban treatment, but previous studies have suggested a possible treatment benefit |**50,51**|.

Key safety issues with direct thrombin inhibitors

The new thrombin inhibitors, in particular ximelagatran, have now been tested in at least 17 000 people in large clinical trials. This experience does not, however, match our knowledge of warfarin, which has been in clinical use for half a century. The main concern regarding the oral direct thrombin inhibitor ximelagatran has been a considerable (>3× upper limit of normal) elevation of plasma concentrations of liver transaminases in approximately 6–7% of people taking the drug and the rather unexpected increased rate of MI in VTE prevention studies. The clinical significance and mechanism behind ximelagatran-induced liver injury is unknown and needs to be defined further before it can be considered for widespread use. For some patients, the liver injury has been quite severe and potentially life-threatening.

The finding that short-term use of ximelagatran might carry an increased risk of acute coronary events was unexpected and rather counterintuitive. *Post hoc* analyses can be misleading and the absolute number of events involved is small. However, this potential adverse effect needs to be prospectively reassessed.

Warfarin has a large number of major limitations and serious adverse effects, and is far from an ideal anticoagulant. Ultimately, as with the liver derangements and rhabdomyolysis with statins, it remains to be established whether the benefits outweigh the risks of therapy with ximelagatran. Advantages and disadvantages of warfarin and ximelagatran are compared in Table 7.4.

Clinical studies of bivalirudin so far indicate that the drug has a very acceptable safety profile with no reported specific adverse effects and no idiosyncratic reactions. There is an inherent risk of bleeding associated with all anticoagulants, but bivalirudin is at least as safe as heparin and it has a short plasma half-life. Because of its partial renal excretion, the dose of bivalirudin should be adjusted in patients with renal impairment.

Table 7.4 Main advantages and disadvantages of warfarin and ximelagatran

Warfarin: advantages	**Ximelagatran: advantages**
Long half-life	Rapid onset of action
Dose can be titrated to required INR	Monitoring of anticoagulation not required
Can be reversed with vitamin K or blood products	No major drug or food interactions
	Predictable pharmacodynamics
	Fixed dosing
Warfarin: disadvantages	**Ximelagatran: disadvantages**
Multiple drug interactions	Deranged liver function tests (~6%)
Food/alcohol interactions	Fixed level of anti-coagulation
Unpredictable pharmacokinetics	Short half-life requiring bid dosing
Slow onset of action	No antidote
Regular and frequent monitoring required	

Source: compiled by present authors.

Conclusions

There are major limitations and problems with established anticoagulant therapies such as warfarin and heparin. The advent of safe, effective and easier to use anticoagulant therapies is needed and welcome. Direct thrombin inhibition is an exciting, potentially important and useful new option in the treatment and prophylaxis of vascular thrombosis. However, many questions remain unanswered and direct thrombin inhibitors have so far only been tested in selected patient groups under clinical trial conditions. Questions of safety have been raised with the first orally active agent, ximelagatran. Anticoagulation levels reached with ximelagatran are fixed and thus might not be sufficient for conditions traditionally requiring higher INR levels such as patients with prosthetic heart valves. The benefits of not having to monitor anticoagulation levels are, at least partly, offset by the need to monitor liver function and increased cost of medications.

The effects of prolonged direct thrombin inhibition are unknown. Thrombin not only acts in the coagulation system but has a widespread physiological role in cell signalling, largely through activation of proteinase-activated receptors. Studies, mainly on small animals, have suggested that thrombin inhibition might reduce tumour invasion and metastases, and be beneficial in inflammatory conditions [**52**]. Whether these potential benefits are likely to extend to adjuvant therapy in patients with cancer or as secondary prevention in patients with coronary heart disease remains to be established.

We are likely to see a major expansion in the indications for, and use of thrombin inhibitors over the next few years and decades. Time will tell whether, and which, new anticoagulants will supersede warfarin and heparin. However, the ease of use, lack of the necessity for anticoagulant monitoring and the apparent low incidence of interactions and side effects, make direct thrombin inhibitors very attractive therapies for patients and clinicians in the fight against vascular diseases.

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Direct Vascular Effects of Protease-Activated Receptor Type 1 Agonism In Vivo in Humans

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Background—Protease-activated receptor type 1 (PAR-1) has been proposed as the principal thrombin receptor in humans, although its actions in vivo have not been defined. The aim of the present study was to determine the direct vascular actions of PAR-1 agonism in humans.

Methods and Results—Dorsal hand vein diameter was measured by the Aellig technique in 14 healthy volunteers during local intravenous SFLLRN (PAR-1 agonist; 0.05 to 15 nmol/min) and SLIGKV (PAR-2 agonist; 1.6 to 160 nmol/min) infusions. The venous effects of SFLLRN were further assessed in the presence or absence of norepinephrine or the glycoprotein IIb/IIIa antagonist tirofiban. Forearm blood flow was measured by venous occlusion plethysmography in 16 volunteers during infusion of SFLLRN (1 to 50 nmol/min), SLIGKV (160 to 800 nmol/min), and the endothelium-dependent vasodilator bradykinin (100 to 1000 pmol/min). Platelet-monocyte binding (a sensitive measure of platelet activation) and plasma tissue plasminogen activator (tPA), plasminogen-activator inhibitor 1, and von Willebrand factor concentrations were measured at intervals throughout the study. SFLLRN caused dose-dependent venoconstriction ($P<0.001$) that was unaffected by norepinephrine or tirofiban co-infusion. In forearm resistance vessels, SFLLRN increased forearm blood flow ($P<0.001$), tPA release ($P<0.001$), and platelet-monocyte binding ($P<0.0001$) without affecting plasma plasminogen-activator inhibitor 1 or von Willebrand factor concentrations. SLIGKV caused venous ($P<0.001$) and arterial ($P<0.01$) dilatation without tPA release.

Conclusions—We have demonstrated that PAR-1 agonism causes platelet activation, venous constriction, arterial dilatation, and tPA release in vivo in humans. These unique and contrasting effects provide important insights into the physiological and pathophysiological role of thrombin in the human venous and arterial circulations. **(*Circulation*. 2006; 114:1625-1632.)**

Key Words: platelets ■ blood flow ■ veins ■ arteries ■ receptor, PAR-1 ■ receptor, PAR-2

Thrombin is a powerful physiological stimulant in the cardiovascular system. Apart from its central enzymatic role in the coagulation cascade, it directly activates platelets, leukocytes, and vascular smooth muscle and endothelial cells.[1] Thrombin is therefore a vital link between thrombosis, cellular activation, and inflammation, key pathogenic factors in atherothrombotic disorders.

Clinical Perspective p 1632

Given the apparent direct cellular effects of thrombin, cloning methods[2,3] were used to conduct an extensive search for its receptor. This led to the identification of G-protein–coupled protease-activated receptors (PARs) characterized by a unique mechanism of activation whereby proteolytic cleavage unmasks a short peptide sequence that remains tethered and activates the receptor.[2,4] Four different types of PARs have been identified. PAR-1, -3, and -4 are all activated by thrombin. The type 1 receptor has been proposed as the principal thrombin receptor in humans.[5,6] In contrast, the PAR-2 receptor is activated by trypsin, by tryptase, and to a lesser extent by coagulation factors upstream of thrombin.[7] It appears to be of importance in inflammatory conditions that induce endothelial PAR-2 expression and vasodilatation.

PAR-1 receptor agonism has been extensively studied in vitro and is associated with platelet activation and aggregation,[8] vasodilatation,[9] and angiogenesis.[10] Although studies in small animals suggest that PAR-1 and PAR-2 agonism induces vasodilatation,[11,12] there is significant species heterogeneity, and rodent models are of limited relevance to humans.[13] Exploring the role of PAR-1 receptors in the human vasculature would deepen our understanding of the physiological role of thrombin and would be of interest in the development of new therapeutic strategies such as PAR-1 receptor antagonists[14,15] and direct thrombin inhibitors.[16,17]

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TABLE 1. Baseline Characteristics of Study Volunteers

Male, %	79 (41/52)
Age, y	24±0.5
Body mass index, kg/m^2	23±0.4
Heart rate, bpm*	67±2
Blood pressure, mm Hg*	
Systolic	132±2
Diastolic	73±1
Hematocrit*	0.42±0.01
White cell count, ×10^9/L*	4.98±0.37
Platelets, ×10^9/L*	213±11

Values are mean±SEM.
*Data from vascular studies only.

Therefore, the aims of the present study were to determine the direct in vivo vascular effects of PAR-1 agonism in the human vasculature. Specifically, we wished to assess the direct role of PAR-1 activation on platelet activation and aggregation, venous and arterial tone, and the release of endothelium-derived factors in vivo in humans.

Methods

Subjects

Fifty-two healthy male and female nonsmokers (age, 20 to 38 years) were recruited into the study (Table 1). Participants had not been taking any regular medications, over-the-counter medications, herbal supplements, or vitamins. They did not have clinically significant coexisting conditions, including hypertension, hyperlipidemia, diabetes mellitus, asthma, and coagulopathy, and had not suffered a recent infective or inflammatory condition. The study was approved by the local research ethics committee and conducted in accordance with the Declaration of Helsinki and with the written informed consent of all volunteers.

Platelet Studies

Protocol 1: Platelet Aggregometry

Blood was sampled with a 19-gauge needle from the antecubital fossa into a 50-mL syringe, transferred into tubes containing 3.8% citrate, and centrifuged at 130*g* for 20 minutes at room temperature to obtain platelet-rich plasma. Blood was centrifuged at 1200*g* for 10 minutes to obtain platelet-poor plasma for reference samples. Platelet aggregation was measured at 37°C using standard optical platelet aggregometry.[18,19]

Preliminary in vitro platelet studies were carried out to construct concentration-response curves to define the concentration of the PAR-1 agonist SFLLRN-NH_2 that caused platelet activation and aggregation. SFLLRN-NH_2 (Auspep, Parkville Victoria, Australia) was compared with the thromboxane A_2 agonist U46619 (Sigma, Gillingham, UK) in both platelet-rich plasma and washed platelets.[18] To assess the specificity of the PAR-1–activating peptide, both SFLLRN-NH_2– and U46619-induced platelet aggregation was assessed in the presence and absence of the PAR-1 antagonist RWJ-58259[13] (αS-*N*-[(1S)-3-amino-1-[[(phenylmethyl)-amino]carbonyl]propyl]-alpha-[[[[[1-(2,6-dichlorophenyl)methyl]-3-(1-pyrrolidinylmethyl)-1H-indazol-6-yl]amino]carbonyl]amino]-3,4-difluorobenzenepropanamide; American Peptide Co, Sunnyvale, Calif) in washed platelets. SFLLRN-NH_2–induced platelet aggregation was also assessed in the presence of the direct thrombin inhibitor lepirudin (0.1 and 1.0 mg/mL; Schering, Burgess Hill, UK).

We chose the concentration of SFLLRN-NH_2 that caused nearly maximal platelet aggregation and assumed that the vascular and platelet PAR-1 receptor sensitivities were equivalent. However, before progressing to in vivo infusions, we defined the dose of tirofiban that would completely inhibit platelet aggregation using the clinical-grade preparation of SFLLRN-NH_2. Platelet-rich plasma was incubated for 5 minutes in the presence or absence of tirofiban (Merck, Sharp and Dohme, Hoddesdon, UK) at 50 ng/mL.[20] Platelet aggregation was measured after administration of 0.06 to 12 μmol/L of the PAR-1–activating peptide SFLLRN-NH_2 (Clinalfa, Laufelfingen, Switzerland) by a 4-channel platelet aggregometry profiler (PAP-4D, Bio/Data Corp, Horsham, Pa) linked to PC interface software (Platelet Aggregation Profiler, version 2.3, Bio/Data Corp).

Protocol 2: Platelet-Monocyte Binding

Venous blood (5 mL) was collected through a 19-gauge needle and transferred into a tube containing the direct thrombin inhibitor D-phenylalanyl-L-propyl-L-arginine chloromethylketone. For in vitro studies, blood was collected into 2 D-phenylalanyl-L-propyl-L-arginine chloromethylketone tubes containing either tirofiban (50 ng/mL) or SFLLRN-NH_2 (0.06 to 6 μmol/L). Five minutes after sampling, blood was incubated with appropriate monoclonal antibodies labeled with fluorochromes for 20 minutes, and platelet monocyte aggregates were measured as described previously.[21]

Vascular Studies

All studies were carried out in a quiet, temperature controlled room (22°C to 24°C). Participants were semirecumbent (venous studies) or supine (arterial studies) and had abstained from alcohol for 24 hours and from food and caffeine-containing drinks for at least 4 hours before the study.

Venous Studies

A 23-gauge needle was sited in a dorsal hand vein, and the total infusion rate was kept constant at 0.25 mL/min in all studies. The hand was supported above the level of the heart and an upper arm cuff inflated to 40 mm Hg to obstruct venous return. The internal diameter of the dorsal hand vein was measured by the Aellig technique.[22] In brief, a magnetized lightweight rod rested on the summit of the infused vein ≈1 cm downstream from the tip of the infusion needle. The rod passed through a core of a linear variable differential transformer, supported above the hand by a small tripod. Changes in vein diameter caused vertical displacement of the rod, leading to a linear change in the voltage generated by the linear variable differential transformer. This enabled calculation of absolute changes in vein size.

Protocol 3: Effect on Venous Tone

Because dorsal hand veins do not have resting tone, norepinephrine (1 to 128 ng/min) was used to induce and maintain a 50% to 70% reduction in vein diameter throughout the study to allow detection of either venodilatation or venoconstriction. This was followed by co-infusion of the PAR-1–activating peptide SFLLRN-NH_2 (0.05 to 5 nmol/min) or the PAR-2–activating peptide SLIGKV-NH_2 (1.6 to 160 nmol/min; Clinalfa).

Protocol 4: Effect of Glycoprotein IIb/IIIa Receptor Antagonism

The effects of SFLLRN-NH_2 (0.05 to 15 nmol/min) in the presence and absence of the glycoprotein IIb/IIIa inhibitor tirofiban (250 ng/min) and norepinephrine (1 to 128 ng/min) were assessed to determine the importance of platelet aggregation on PAR-1–mediated alterations of venous tone. The doses of tirofiban and SFLLRN-NH_2 were chosen to achieve end-organ concentrations equivalent to those with efficacy in in vitro studies (protocol 1) and assumed a dorsal hand vein flow of 5 mL/min.

Arterial Studies

All subjects underwent brachial artery cannulation with a 27-standard-wire-gauge steel needle under controlled conditions. Intra-arterial infusion rate was kept constant at 1 mL/min throughout all studies. Forearm blood flow was measured in the infused and noninfused arms by venous occlusion plethysmography using mercury-in-Silastic strain gauges as described previously.[23,24] Supine heart rate and blood pressure were monitored at intervals throughout each study using a semiautomated noninvasive oscillometric sphygmomanometer. Tirofiban (1.25 μg/min) was co-infused during PAR-1 activation to inhibit in vivo potential platelet aggre-

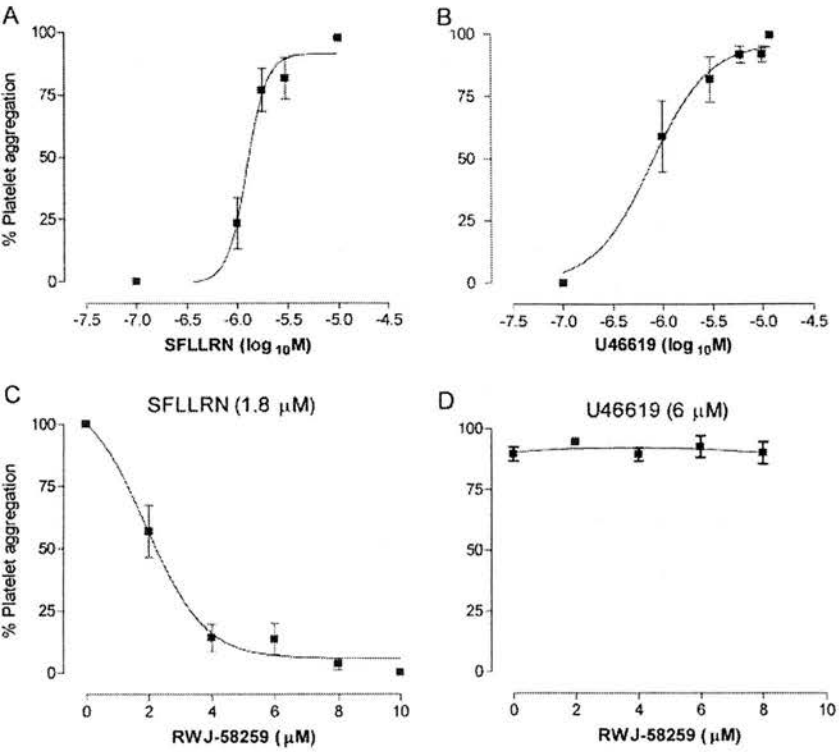

Figure 1. Induction of platelet aggregation by the PAR-1–activating peptide SFLLRN-NH_2 (A) and the thromboxane A_2 agonist U46619 (B; $P<0.001$ for both, 1-way ANOVA). The PAR-1 antagonist RWJ-58259 inhibited aggregation induced by SFLLRN-NH_2 (C; $P<0.001$, 1-way ANOVA) but not by U46619 (D; P=NS). Values are mean±SEM.

gation. The doses of tirofiban and SFLLRN-NH_2 were chosen to achieve end-organ concentrations equivalent to those with efficacy in in vitro studies (protocol 1) and assumed a brachial artery blood flow of 25 mL/min.

Blood Sampling

Venous cannulas (17 gauge) were inserted bilaterally into the antecubital fossae. Blood samples were drawn simultaneously from each arm during infusion of saline, tirofiban, and each dose of the PAR-activating peptides and bradykinin. They were collected into acidified buffered citrate (Stabilyte, Trinity Biotech Plc, Co Wicklow, Ireland; for tissue plasminogen activator [tPA] assays) and into citrate (BD Vacutainer, BD UK Ltd, Oxford, UK; for plasminogen activator inhibitor type 1 [PAI-1] and von Willebrand factor [vWF] assays). Samples were kept on ice before centrifugation at 2000*g* for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at −80°C before assay. Plasma tPA antigen and activity (tPA Combi Actibind Elisa Kit, Technoclone, Vienna, Austria) and PAI-1 antigen and activity (ElitestPAI-1 Antigen and Zymutest PAI-1 Activity, Hyphen Biomed, Neuville-Sur-Oise, France) concentrations were determined by ELISAs. Full blood count was measured at baseline and at the end of the study. Blood was also collected from each arm to determine platelet-monocyte binding (see protocol 2) at baseline, at the highest dose of SFLLRN-NH_2, during saline washout, and at the highest dose of bradykinin.

Protocol 5: PAR-1 Activation

After a 20-minute baseline saline infusion, tirofiban (1.25 μg/min) was infused throughout the study. Thirty minutes after the tirofiban infusion was started, the PAR-1–activating peptide SFLLRN-NH_2 was co-infused at 5, 15, and 50 nmol/min for 8 minutes at each dose separated by 6 minutes of saline washout infusions. This was followed by a 30-minute washout period before co-infusion of bradykinin (an endothelium-dependent vasodilator that releases tPA; Clinalfa) at 100, 300, and 1000 pmol/min[25] for 8 minutes at each dose.

Protocol 6: PAR-2 Activation

After a 20-minute baseline saline infusion, the PAR-2–activating peptide SLIGKV-NH_2 was infused at 160, 360, and 800 nmol/min for 8 minutes at each dose separated by 6 minutes of saline washout infusions. This was followed by a 30-minute saline infusion before bradykinin was infused at 100, 300, and 1000 pmol/min[25] for 8 minutes at each dose.

Data and Statistical Analyses

Dorsal hand venous[26] and forearm plethysmographic[23] data were analyzed as described previously. Variables are reported as mean±SEM and analyzed using repeated-measures 1- or 2-way ANOVA with post-hoc Bonferroni corrections and 2-tailed Student *t* test as appropriate. Statistical analysis was performed with GraphPad Prism (GraphPad Software, Inc, San Diego, Calif). Statistical significance was taken at $P<0.05$.

The authors had full access to the data and take responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results

In Vitro Effects of PAR Agonism on Platelets

Both SFLLRN-NH_2 and U46619 caused dose-dependent platelet aggregation (median effective concentration [EC_{50}]=1.26 and 0.78 μmol/L, respectively; Figure 1A and 1B; n=8). The PAR-1 antagonist RWJ-58259 selectively and fully inhibited SFLLRN-NH_2–induced (Figure 1C; n=8; $P<0.001$, 1-way ANOVA) but not U46619-induced (Figure 1D; n=8; P=NS) platelet aggregation. In contrast, the direct thrombin inhibitor lepirudin had no effect on SFLLRN-NH_2–induced platelet aggregation (n=6; P=NS; data not shown).

The clinical-grade SFLLRN-NH_2 also caused a dose-dependent increase in platelet aggregation (EC_{50}=0.66 μmol/L) that was inhibited by tirofiban (Figure 2A; n=6; $P<0.001$, 2-way ANOVA). In contrast, PAR-1 activation caused a dose-dependent increase in whole-blood platelet-monocyte binding (EC_{50}=0.23 μmol/L) that was not affected by tirofiban (Figure 2B; n=6; P=NS, 2-way ANOVA). As anticipated, PAR-2

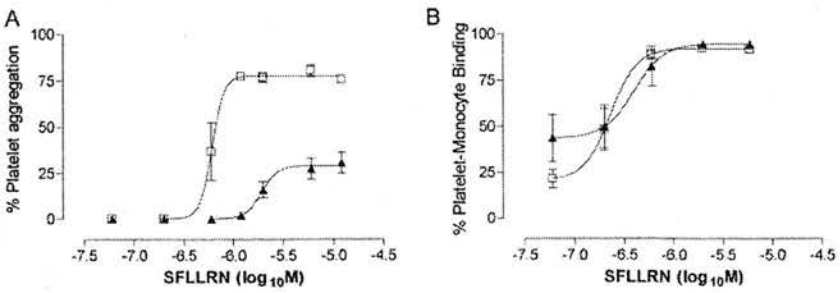

Figure 2. PAR-1–activating peptide (SFLLRN-NH_2) induced platelet aggregation (A) and platelet-monocyte binding (B) in the presence (▲) or absence (□) of tirofiban (50 ng/mL). Values are mean±SEM. A, $P<0.0001$ (2-way ANOVA) vs tirofiban; B, P=NS (2-way ANOVA) vs tirofiban.

activation with SLIGKV-NH_2 induced neither platelet aggregation nor platelet-monocyte binding (n≥3; data not shown).

In Vivo Effects of PAR Agonism on Dorsal Hand Veins

After venoconstriction (50% to 70%) was induced and maintained with norepinephrine, SFLLRN-NH_2 and SLIKGV-NH_2 caused dose-dependent venoconstriction (n=8; $P<0.001$, 1-way ANOVA) and venodilatation (n=6; $P<0.001$, 1-way ANOVA), respectively. In the absence of norepinephrine, SFLLRN-NH_2 caused a dose-dependent venoconstriction ($P<0.001$, 1-way ANOVA) that was able to induce complete constriction of the venous segment. This was not mediated by platelet aggregation because co-infusion of tirofiban had no effect on SFLLRN-NH_2–induced venoconstriction (Figure 3). SFLLRN-NH_2 was well tolerated by all subjects with no adverse effects. Vein patency was maintained at all times with no clinically apparent in situ thrombus formation.

In Vivo Effects of PAR Agonism on Forearm Resistance Vessel Tone

There was no change in heart rate, blood pressure, or noninfused forearm blood flow throughout either study. Intra-arterial tirofiban had no effect on resting forearm blood flow (P=NS). Both PAR-1 activation with SFLLRN-NH_2 and PAR-2 activation with SLIGKV-NH_2 caused dose-

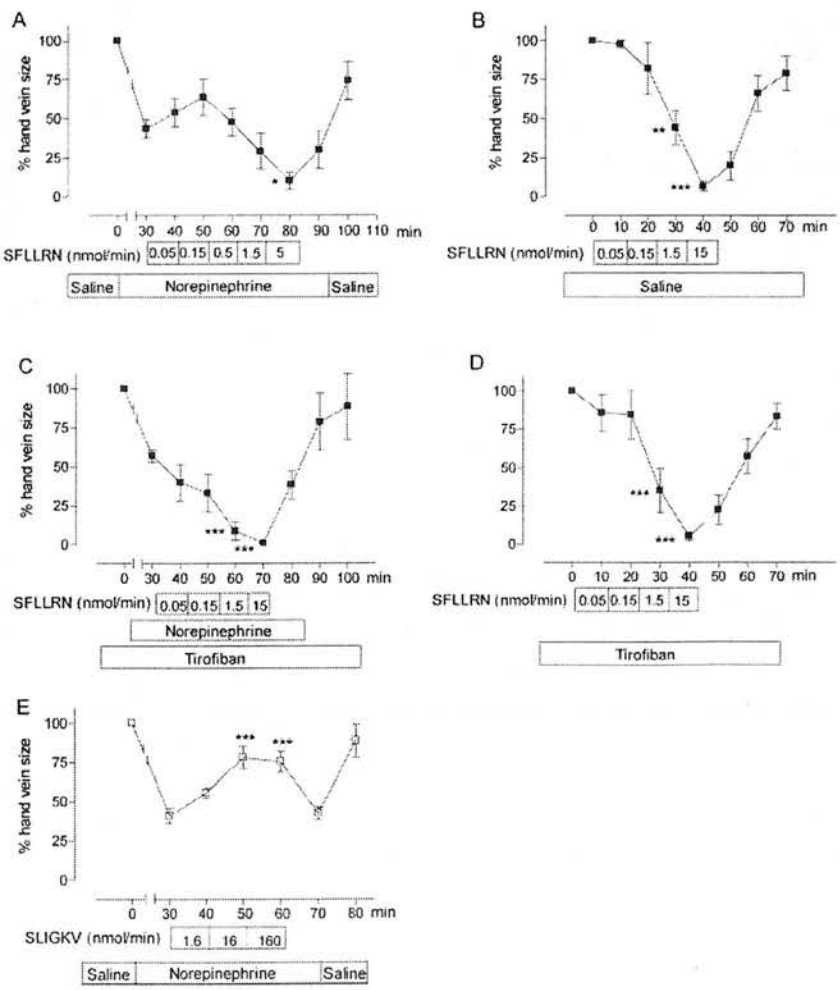

Figure 3. The PAR-1–activating peptide SFLLRN-NH_2 causes concentration-dependent venoconstriction (■; n=6 to 8; $P<0.001$, 1-way ANOVA) that is unaffected by norepinephrine (A, B) or tirofiban (C, D) infusion, whereas the PAR-2–activating peptide SLIGKV-NH_2 (E) induces venodilatation (□; n=6; $P<0.001$, 1-way ANOVA). Values are mean±SEM. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

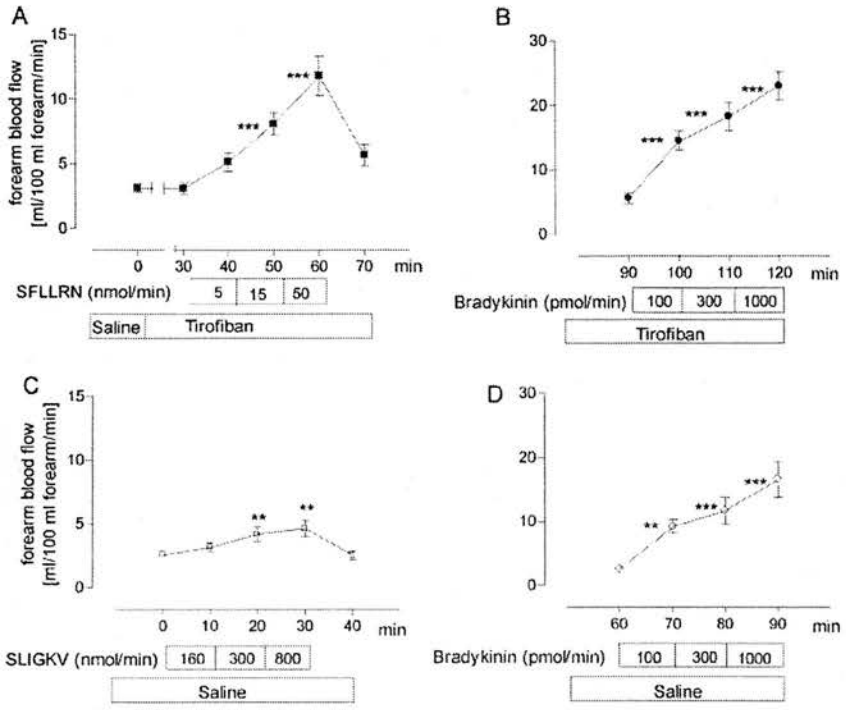

Figure 4. Forearm vasodilatation induced by the PAR-1–activating peptide SFLLRN-NH_2 (A; ■; n=8; $P<0.001$, 1-way ANOVA), the PAR-2–activating peptide SLIGKV-NH_2 (C; □; n=8; $P<0.01$, 1-way ANOVA), and bradykinin (B, ●; D, ○; n=8; $P<0.001$, 1-way ANOVA). Values are mean±SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

dependent vasodilatation (Figure 4A and 4C; n=8; $P<0.001$ and $P<0.01$, respectively, 1-way ANOVA) with a rapid onset and offset of action. As anticipated, bradykinin increased forearm blood flow (Figure 4B; $P<0.001$, 1-way ANOVA) and was unaffected by tirofiban co-infusion (Figure 4D; P=NS versus no tirofiban).

In Vivo Effects of PAR Agonism on Endothelium-Derived Factors

Plasma tPA antigen and activity concentrations increased in a dose-dependent manner during SFLLRN-NH_2 and bradykinin but not SLIGKV-NH_2 infusion (Figure 5; $P<0.001$ for all). Plasma PAI-1 antigen and activity and vWF concentrations were unaffected by all infusions (P=NS for all; Table 2). Although there was an apparent rise in plasma PAI-1 antigen concentration at 50 nmol/min SFLLRN-NH_2, it did not achieve statistical significance ($P=0.08$, paired t test versus baseline) and was not associated with increases in PAI-1 activity or vWF. There were no differences in peripheral blood hematocrit (0.416±0.006 versus 0.426±0.008) or platelet counts ($213\pm16\times10^9$ versus $216\pm15\times10^9$/L) at baseline and the end of the study. SFLLRN-NH_2 increased in

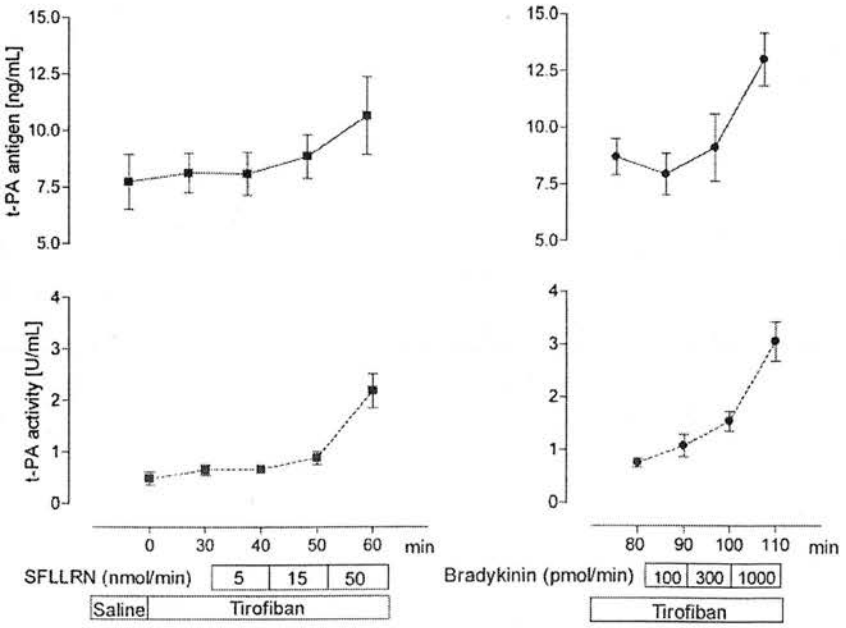

Figure 5. Plasma tPA antigen (solid lines) and activity (dashed lines) during infusion of the PAR-1–activating peptide SFLLRN-NH_2 (■) and bradykinin (●). Values are mean±SEM.

TABLE 2. Plasma PAI-1 Antigen and Activity and vWF Antigen Concentrations in the Infused and Noninfused Arms During Brachial Artery Infusion of SFLLRN-NH_2 and Bradykinin

	PAI-1 Antigen, ng/mL		PAI-1 Activity, U/mL		vWF Antigen, ng/mL	
	Infused Arm	Noninfused Arm	Infused Arm	Noninfused Arm	Infused Arm	Noninfused Arm
Baseline	27.3±5.5	27.7±5.6	0.58±0.14	0.51±0.13	0.72±0.08	0.72±0.07
Tirofiban	25.6±5.5	24.5±4.4	0.40±0.09	0.43±0.12	0.69±0.04	0.62±0.08
SFLLRN-NH_2, nmol/min						
5	23.0±4.7	24.9±4.4	0.44±0.12	0.47±0.11	0.62±0.06	0.67±0.08
15	24.3±4.5	23.8±4.4	0.41±0.10	0.41±0.11	0.67±0.06	0.74±0.10
50	33.8±6.8	26.9±5.3	0.37±0.09	0.42±0.09	0.73±0.08	0.76±0.08
Washout	22.9±4.2	23.1±3.6	0.32±0.08	0.33±0.08	0.67±0.09	0.74±0.09
Bradykinin, pmol/min						
100	19.6±3.7	21.3±3.9	0.27±0.07	0.35±0.09	0.80±0.08	0.64±0.09
300	19.5±4.4	19.5±4.9	0.25±0.06	0.26±0.07	0.73±0.08	0.70±0.07
1000	19.5±3.2	19.1±2.9	0.16±0.03	0.25±0.05	0.76±0.07	0.67±0.07

n=8 for all. Values are mean±SEM.

vivo platelet-monocyte binding (from 16±5% to peak of 74±5%; Figure 6; $P<0.0001$, 1-way ANOVA) that declined toward baseline with time.

The PAR-2–activating peptide SLIGKV-NH_2 had no effect of plasma tPA concentrations (P=NS for all; data not shown).

Discussion

In a series of clinical studies, we have, for the first time, described the direct vascular effects of PAR-1 agonism and established the induction of in vivo platelet activation, venoconstriction, vasodilatation, and tPA release, as well as confirming the vasodilatory effects of PAR-2 agonism. These unique and contrasting effects of PAR-1 activation provide important insights into the physiological and pathophysiological roles of thrombin in the venous and arterial circulations of humans.

Platelet Effects of PAR-Activating Peptides

Inhibition of platelet activation has been of major benefit in the prevention and treatment of cardiovascular diseases. Thrombin is one of the most potent physiological stimulants of platelet activation that appears to be mediated largely through the PAR-1 receptor, although major contributions are also provided by PAR-4 and glycoprotein Ib receptors. To date, strategies to reduce the action of thrombin have focused on direct inhibition of its enzymatic activity. Recently, PAR-1 antagonists have been developed as potential antiplatelet agents[15] that do not affect the coagulation cascade. It is therefore important to define the in vitro and in vivo effects of PAR-1 agonism in humans. As anticipated, we have demonstrated that PAR-1 but not PAR-2 agonism caused concentration-dependent platelet activation and aggregation. Moreover, in the presence of complete inhibition of platelet aggregation with the glycoprotein IIb/IIIa receptor antagonist tirofiban, PAR-1 agonism continued to induce platelet activation as measured by platelet-monocyte binding both in vitro and in vivo. This is consistent with our previous findings that although glycoprotein IIb/IIIa receptor antagonism inhibits platelet aggregation, it does not affect platelet activation or platelet-monocyte binding.[27]

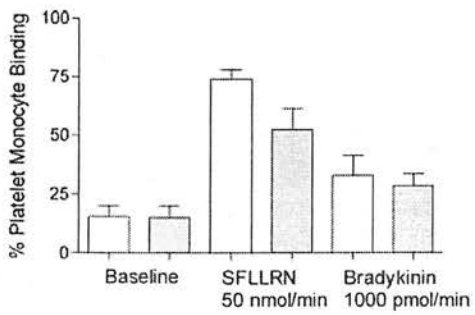

Figure 6. Platelet-monocyte binding during the infusion of the PAR-1 agonist SFLLRN-NH_2 in the infused (open bars) and noninfused (gray bars) arms. Values are mean±SEM.

Vascular Effects of PAR-Activating Peptides

PAR-1 appears to be the principal receptor responsible for the vascular actions of thrombin and includes the regulation of vasomotion, platelet aggregation, inflammation, and angiogenesis. However, investigation of the in vivo vascular effects of thrombin presents challenges because of the direct activation of the coagulation cascade and platelets that may result in intravascular thrombosis. Here, we have chosen to use PAR-activating peptides that cause direct cellular stimulation without enzymatic activation of the coagulation cascade.

The direct vascular effects of PAR-1 receptor agonism have not been previously reported in humans. Our study protocol was vigilant to minimize any potential prothrombotic effects associated with PAR-1–induced platelet aggregation. We defined the in vitro concentrations of glycoprotein IIb/IIIa receptor antagonism that would permit the direct in vivo intravascular infusion of PAR-1–activating peptide without inducing platelet aggregation and potential thrombosis. This appears to have been successfully achieved, given the absence of significant adverse clinical side effects or thrombosis.

Venous Effects of PAR-Activating Peptides

Thrombin and the PAR-1 agonist SFLLRN-NH_2 have previously been shown to cause endothelium- and nitric oxide–dependent venous and arterial dilatation in canine and porcine ring segments.[28,29] Although confirming previous observations of PAR-2–mediated venodilatation,[30] we have rather unexpectedly described a dose-dependent venoconstriction with PAR-1 activation. This may be mediated by platelet activation, release of endothelium-derived vasoconstrictors, or a direct effect on venous smooth muscle cells. Neither norepinephrine nor tirofiban co-infusion appeared to affect this potent venoconstrictor effect. In combination with the apparent absence of in situ thrombosis, this suggests that PAR-1–induced venoconstriction was not mediated by platelet aggregation, although we cannot exclude an effect of platelet activation.

Arterial Effects of PAR-Activating Peptides

In contrast to the venous effects, both PAR-1 and PAR-2 activation caused dose-dependent vasodilatation of forearm resistance vessels. We have not assessed the mechanism of this vasodilatation, but animal and clinical models suggest that this is likely to be endothelium and nitric oxide dependent.[11,30] However, data from animal studies have limited relevance in humans because of the wide species variability in PAR-1 receptor expression and function. The mechanism of PAR-1–induced vasodilatation needs to be addressed in future clinical studies.

PAR receptor expression varies between endothelial cell cultures originating from different human blood vessels,[31] which may partly explain the contrasting responses between PAR-1 and PAR-2 in the arterial and venous circulations. Our findings that PAR-2 activation causes venous and arterial dilatation are consistent with previous findings[30] and support the vascular role of PAR-2 receptors in inflammatory conditions.

Effects of PAR-Activating Peptides on Endothelium-Derived Factors

Intra-arterial PAR-1 but not PAR-2 agonism caused an acute dose-dependent increase in local endothelial tPA release. In contrast to in vitro human endothelial culture studies,[32] this occurred in the absence of the release of other endothelium-derived factors such as PAI-1 and vWF. However, there was an apparent rise in plasma PAI-1 antigen ($P=0.08$) with high-dose PAR-1 agonism, but given the absence of an effect on PAI-1 activity and vWF, this may reflect the associated platelet activation and release of platelet-derived PAI-1. PAI-1 is stored in platelet α-granules, where its activity is $\approx$5% of plasma because of the absence of the stabilizing effect of vitronectin. Thus, PAR-1 agonism appears to have a selective profibrinolytic effect on the arterial endothelium.

Physiological Significance of Vascular PAR-1 Activation

We have, for the first time, described the unexpected and contrasting vascular effects of PAR-1 agonism in vivo in humans. How do we interpret these effects? In an intact normal vessel, homeostatic mechanisms attempt to maintain vessel patency and minimize intravascular thrombus formation. Dorsal hand veins do not have resting tone, and the induction of venodilatation will not affect venous blood flow. Therefore, in the presence of developing venous thrombosis, venodilatation would not be beneficial, whereas venoconstriction will potentially limit thrombus propagation and embolization. In contrast, it would be anticipated that increasing blood flow and endogenous fibrinolysis would limit arterial thrombosis by ensuring rapid clearance and dissolution of a developing thrombus. We therefore propose that the vascular effects of PAR-1 agonism in vivo in humans can be understood in terms of limiting intravascular thrombosis and maintaining vessel patency. We speculate that these physiological effects may be disturbed in patients with cardiovascular disease or prothrombotic disorders.

Conclusions

PAR-1 agonism causes platelet activation, venoconstriction, vasodilatation, and tPA release in vivo in humans. This has important implications in our understanding of the physiological vascular effects of thrombin and the pathogenesis of thromboembolic and atherothrombotic disorders.

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Disclosures

None.

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CLINICAL PERSPECTIVE

In addition to its well-known central enzymatic role within the coagulation cascade, thrombin is a powerful direct cellular activator of platelets, leukocytes, and vascular smooth muscle and endothelial cells. It is therefore a vital link between thrombosis and inflammation, key pathogenic factors in atherothrombotic disease. It has recently been discovered that thrombin causes direct cellular activation through a group of G-protein–coupled protease-activated receptors (PARs). Although the PAR-1 receptor is believed to mediate most of the receptor-mediated effects of thrombin, the cellular actions of PAR-1 activation have not been described in vivo in humans. In the present study, we establish for the first time the in vivo vascular effects of PAR-1 activation and demonstrate that it causes venous constriction, arterial dilatation, platelet activation, and release of endogenous tissue plasminogen activator. These intriguing and contrasting effects of PAR-1 agonism demonstrate the diverse nature of the vascular consequences of thrombin activation in humans. We suggest that, in the presence of a developing intravascular thrombosis, venoconstriction would limit thrombus propagation and embolization, whereas in the arterial circulation, increasing blood flow and endogenous fibrinolysis would limit arterial thrombosis by ensuring thrombus clearance and dissolution. We therefore propose that the vascular effects of PAR-1 agonism in vivo in humans can be understood in terms of limiting intravascular thrombosis and maintaining vessel patency. This has potential implications for applying novel PAR-1 receptor antagonists that are currently under clinical development as potential antiplatelet therapies.

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Role of the Endothelium in the Vascular Effects of the Thrombin Receptor (Protease-Activated Receptor Type 1) in Humans

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Objectives The purpose of this study was to determine the role of the endothelium in the vascular actions of protease-activated receptor type 1 (PAR-1) activation in vivo in man.

Background Thrombin is central to the pathophysiology of atherothrombosis. Its cellular actions are mediated via PAR-1. Protease-activated receptor type 1 activation causes arterial vasodilation, venoconstriction, platelet activation, and tissue-type plasminogen activator release in man.

Methods Dorsal hand vein diameter was measured in 6 healthy volunteers before and after endothelial denudation. Forearm arterial blood flow, plasma fibrinolytic factors, and platelet activation were measured in 24 healthy volunteers during venous occlusion plethysmography. The effects of inhibition of prostacyclin, nitric oxide (NO), and endothelium-derived hyperpolarizing factor on PAR-1 responses were assessed during coadministration of aspirin, the "NO clamp" (L-N^G-monomethyl arginine and sodium nitroprusside), and tetraethylammonium ion, respectively.

Results Endothelial denudation did not affect PAR-1–evoked venoconstriction (SFLLRN; 0.05 to 15 nmol/min). Although aspirin had no effect, SFLLRN-induced vasodilation (5 to 50 nmol/min) was attenuated by the NO clamp ($p < 0.0001$) and tetraethylammonium ion ($p < 0.05$) and abolished by their combination ($p < 0.01$). The NO clamp augmented SFLLRN-induced tissue-type plasminogen activator and plasminogen activator inhibitor type 1 antigen ($p < 0.0001$) release, but tetraethylammonium ion and aspirin had no effect. SFLLRN-induced platelet activation was unaffected by NO or prostacyclin inhibition.

Conclusions Acting via PAR-1, thrombin causes contrasting effects in the human vasculature and has a major interaction with the endothelium. This highlights the critical importance of endothelial function during acute arterial injury and intravascular thrombosis, as occurs in cardiovascular events including myocardial infarction and stroke. (J Am Coll Cardiol 2008;51:1749–56) © 2008 by the American College of Cardiology Foundation

Thrombin plays a central role in the coagulation cascade and thrombosis (1). It is one of the most powerful physiological agonists in the cardiovascular system, and its actions are fundamental to the processes of atherosclerosis and its thrombotic consequences.

In addition to the enzymatic generation of fibrin, thrombin stimulates a range of cell types including platelets, endothelial cells, and vascular smooth muscle cells. An extensive search for thrombin receptors ultimately culminated in the identification of a group of G-protein coupled receptors termed protease-activated receptors (PARs).

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These receptors are characterized by a unique mechanism of activation whereby the receptor undergoes proteolytic cleavage, unmasking a short peptide sequence that remains tethered and auto-activates the receptor (2,3). To date, 4 different types of PARs have been identified: PAR-1, -3, and -4 are all activated by thrombin; PAR-2 is mainly activated by trypsin but transactivation of PAR-2 by cleaved PAR-1 has been recognized (4,5).

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Abbreviations and Acronyms

ANOVA = analysis of variance
EDHF = endothelium-derived hyperpolarizing factor
L-NMMA = L-N^G-monomethyl arginine citrate
NO = nitric oxide
PAI-1 = plasminogen activator inhibitor type 1
PAR = protease-activated receptor
PGI_2 = prostacyclin
SNP = sodium nitroprusside
TEA = tetraethylammonium ion
t-PA = tissue-type plasminogen activator
vWF = von Willebrand factor

Protease-activated receptor type 1 receptors are the principal thrombin receptors in man and extensive studies in small animals and cell cultures suggest that they have a diverse and important role in various organs. Their activation stimulates a network of G-protein coupled signaling pathways that involve phospholipase Cβ, protein kinase C, calcium release, mitogen-activated protein kinases, and potassium channels (6,7). However, there is significant species heterogeneity with pre-clinical studies of limited relevance to man (8). Exploring the role of PAR-1 receptors in the human vasculature would deepen our understanding of the physiological role of thrombin and be important in the clinical development of new therapeutic strategies.

To understand the physiological actions of thrombin in the uman vasculature is challenging because direct thrombin nstillation has the potential to cause acute thrombosis in itu and hence vascular occlusion. The use of a PAR-1 eceptor agonist, however, permits the direct assessment of ellular responses to thrombin without the enzymatic activation of the coagulation cascade and fibrin formation. Using the short peptide mimetic SFLLRN, we have recently described the in vivo effects of PAR-1 activation in latelets, endothelium and vascular smooth muscle in man. For the first time, we were able to show that PAR-1 ctivation has unique and contrasting effects in the human asculature including arterial dilation, venous constriction, latelet activation, and tissue-type plasminogen activator t-PA) release (9). Given the central role of thrombin in the athophysiology of cardiovascular disease, it is important to stablish the mechanisms of these PAR-1–mediated effects nd, in particular, the role of the endothelium. We therefore et out to explore the role of the endothelium in the vascular ctions of PAR-1 activation in vivo in man.

Methods

ubjects. A total of 30 healthy nonsmokers (mean age 22 ears; range 19 to 37 years) were recruited into the study. The study was approved by the local research ethics committee and conducted in accordance with the Declaration of Helsinki and with the written informed consent of all olunteers. Participants were screened and excluded for linically significant conditions including hypertension, hyerlipidemia, diabetes mellitus, asthma, and coagulopathy. No participant had suffered a recent infective or inflammatory condition or had taken any medications in the 7 days prior to the study.

Vascular assessments. All studies were carried out in a quiet, temperature-controlled room (22°C to 24°C). Participants were semirecumbent (venous studies) or supine (arterial studies) and had abstained from alcohol for 24 h and from food and caffeine-containing drinks for at least 4 h prior to the study.

Venous studies. A 23-G needle was sited in a dorsal hand vein and total infusion rate kept constant at 0.25 ml/min in all studies. The hand was supported above the level of the heart and an upper arm cuff inflated to 40 mm Hg to obstruct venous return. The internal diameter of the dorsal hand vein was measured by the Aellig technique (10) in 6 healthy volunteers. In brief, a magnetized lightweight rod rested on the summit of the infused vein approximately 1 cm downstream from the tip of the infusion needle. The rod passes through the core of a linear variable differential transformer supported above the hand by a small tripod. Changes in diameter of the vein caused vertical displacement of the rod, leading to a linear change in the voltage generated by the transformer. This enabled calculation of absolute changes in vein size.

PROTOCOL 1. VENOUS EFFECTS OF PAR-1 ACTIVATION. First, we established the presence of functional endothelium. As dorsal hand veins do not have resting tone, norepinephrine (1 to 128 ng/min) was used to induce 70% reduction in vein diameter. Once stable venoconstriction was obtained, acetylcholine (1 nmol/min; Novartis Pharmaceuticals UK Ltd, Frimley, United Kingdom) was coinfused with norepinephrine for 8 min to demonstrate endothelium-dependent venodilation and an intact, functional endothelium. Following a 20-min saline infusion, the PAR-1 activating peptide, SFLLRN-NH_2 (0.05 to 15 nmol/min; Clinalfa AG, Laufelfingen, Switzerland), was administered intravenously before a final 20-min saline washout infusion.

PROTOCOL 2. VENOUS EFFECTS OF PAR-1 ACTIVATION FOLLOWING ENDOTHELIAL DENUDATION. At the end of protocol 1, the endothelium of the venous segment was denuded as previously described (11). In brief, a second 23-G butterfly needle was sited 3 to 4 cm downstream from the tip of the infusion-needle, and this segment of the vein was isolated by the use of occlusion wedges. Distilled water was infused through the venous segment at a rate of 5 ml/min for 15 min, thereby causing endothelial denudation that persists for at least 2 days (11). Aspirin (300 mg orally) was given 30 min prior to start of the first study and on each of the 2 subsequent days to prevent venous thrombosis. On the second day following denudation, subjects reattended and protocol 1 was repeated.

Arterial studies. All subjects underwent cannulation of the brachial artery with a 27-G standard wire steel needle under controlled conditions. The intra-arterial infusion rate was kept constant at 1 ml/min throughout all studies. Forearm blood flow was measured in the infused and noninfused arms by venous occlusion plethysmography using mercury-in-

Silastic strain gauges as described previously (12,13). Supine heart rate and blood pressure were monitored at intervals throughout each study using a semiautomated noninvasive oscillometric sphygmomanometer. Tirofiban (1.25 μg/min) was coinfused during the studies to inhibit potential PAR-1 activation-induced platelet aggregation in vivo (9). This dose of tirofiban does not affect platelet-monocyte binding, forearm blood flow, or baseline concentration of t-PA (9).

PROTOCOL 3: ROLE OF NITRIC OXIDE AND PROSTACYCLIN IN PAR-1–INDUCED VASODILATION. Forearm blood flow was measured by venous occlusion plethysmography in response to brachial artery infusion of SFLLRN (PAR-1 agonist; 5 to 50 nmol/min) with tirofiban (1.25 μg/min) in 8 healthy volunteers on 4 visits using a randomized controlled crossover study employing a 2-by-2 factorial design: with and without aspirin (600 mg orally; to inhibit prostacyclin [PGI_2] synthesis) and the "nitric oxide (NO) clamp." Assuming total forearm blood flow of 25 ml/min, this will achieve end-organ concentrations of 0.2 to 2.0 μmol/l SFLLRN.

The NO clamp was used to determine the contribution of NO in PAR-1–mediated vascular effects. Following baseline intra-arterial tirofiban infusion, the NO synthase inhibitor, L-N^G-monomethyl arginine citrate (L-NMMA; 8 μmol/min), was coinfused. To compensate for L-NMMA-induced basal vasoconstriction, forearm blood flow was returned to baseline using a titrated dose of exogenous NO in the form of intrabrachial sodium nitroprusside (SNP; 90 to 900 ng/min). This dose of SNP was coinfused with L-NMMA and continued throughout the study. This arrangement allows a constant "clamped" delivery of exogenous NO while endogenous NO synthase activity is inhibited.

PROTOCOL 4: ROLE OF CALCIUM-ACTIVATED POTASSIUM CHANNELS/ENDOTHELIUM-DERIVED HYPERPOLARIZING FACTOR IN PAR-1–INDUCED VASODILATION. Forearm blood flow was measured in 8 other healthy volunteers in whom intrabrachial SFLLRN (5 to 50 nmol/min), bradykinin (30 to 300 pmol/min), and SNP (2 to 8 μg/min) were coinfused with either saline placebo or tetraethylammonium ion (TEA) (1 mg/min) on either of 2 visits using a randomized double-blind crossover design. Again, agonists were coinfused with intra-arterial tirofiban (1.25 μg/min), which was continued throughout the study. At the dose used, TEA is a nonselective potassium channel antagonist (14–16).

PROTOCOL 5: ROLE OF ENDOTHELIUM-DEPENDENT VASODILATORS IN PAR-1–INDUCED VASODILATION. In the final series of studies, TEA or saline placebo was coinfused with ascending doses of bradykinin and SFLLRN in 8 volunteers using a randomized double-blind crossover design. In this series, endothelium-derived hyperpolarizing factor (EDHF) activity was isolated by inhibiting NO and PGI_2 production on both visits. The NO clamp was employed as described previously (protocol 3), and cyclooxygenase activity was inhibited with a single 600-mg dose of oral aspirin 1 h before each study.

BLOOD SAMPLING. Seventeen-gauge venous cannulae were inserted into left and right antecubital fossae. Blood samples were drawn simultaneously from each arm at baseline. Blood samples were also drawn before SFLLRN or bradykinin infusion and after each dose of SFLLRN or bradykinin. Blood was collected into acidified buffered citrate (Stabilyte, Biopool International, Umeå, Sweden; for t-PA assays) and into citrate (BD Vacutainer, BD UK Ltd, Oxford, United Kingdom; for plasminogen activator inhibitor type 1 [PAI-1], beta thromboglobulin, and von Willebrand factor [vWF] assays). Samples were kept on ice before centrifugation at 2,000 g for 30 min at 4°C. Platelet-free plasma was decanted and stored at −80°C before assay. Plasma t-PA antigen and activity (t-PA Combi Actibind Elisa Kit, Technoclone, Vienna, Austria), PAI-1 antigen and activity (Elitest-PAI-1 Antigen and Zymutest PAI-1 Activity, HYPHEN BioMed, Neuville-sur-Oise, France), beta thromboglobulin (Asserachrom Btg, Diagnostica Stago, Asnières sur Seine, France), and vWF (Dako, Glostrup, Denmark) concentrations were determined by enzyme-linked immunosorbent assays. Full blood count was measured at baseline and at the end of the study.

PLATELET-MONOCYTE BINDING. In protocols 3 and 4, blood was collected from each arm for determination of platelet-monocyte binding at baseline and after the highest dose of SFLLRN. Samples of 5 ml of venous blood were collected and transferred into a tube containing the direct thrombin inhibitor, D-phenylalanyl-L-propyl-L-arginine chloromethylketone. Five minutes after sampling, blood was incubated with appropriate monoclonal antibodies labeled with fluorochromes for 20 min and platelet-monocyte aggregates measured as described previously (17).

Data analysis and statistics. Dorsal hand venous (18) and forearm plethysmographic (12) data were analyzed as described previously. Variables are reported as means ± SEM and analyzed using repeated measures analysis of variance (ANOVA) with post hoc Bonferroni corrections and 2-tailed Student *t* test as appropriate. Statistical analysis was performed with GraphPad Prism (Graph Pad Software, San Diego, California) and statistical significance taken at the 5% level. The authors had full access to the data and take responsibility for its integrity. All authors have read and agreed to the manuscript as written.

Results

Endothelium and PAR-1–induced venoconstriction. The role of the endothelium in PAR-1–induced vasomotor effects was assessed by comparing venous responses before and after local endothelial denudation. This was achieved through brief instillation of distilled water in an isolated dorsal hand vein segment. After pre-constriction with norepinephrine, the presence or absence of functional endothelium was confirmed by the coinfusion of acetylcholine (1 nmol/min). Acetylcholine caused venodilation in the presence of endothelium and venoconstriction in its absence

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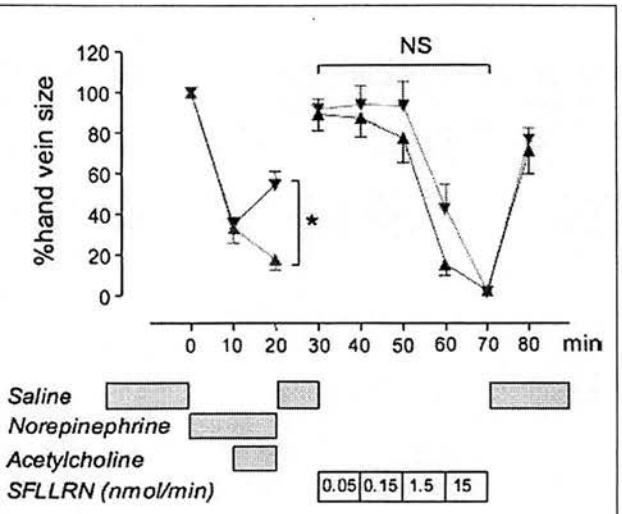

Figure 1 **Dorsal Hand Vein Responses to Acetylcholine and SFLLRN (After Pre-Constriction With Norepinephrine)**

Before **(blue triangles)** and after **(red triangles)** endothelial denudation. *p < 0.05; NS = nonsignificant (p = 0.09); presence versus absence of the endothelium (analysis of variance).

Fig. 1) (from 35 ± 4% to 55 ± 7% in the presence of ndothelium versus 33 ± 7% to 18 ± 6% in the absence of ndothelium; $p < 0.01$ for both; ANOVA). After endothe-ial denudation, there appeared to be a trend toward nhanced venoconstriction induced by the PAR-1 activat-ng peptide, SFLLRN (Fig. 1) ($p = 0.09$; ANOVA).

Endothelium-derived vasodilators and PAR-1–induced vasodilation. SFLLRN caused an increase in forearm lood flow that was unaffected by PGI_2 inhibition with oral spirin (600 mg) (Fig. 2A). The NO synthase inhibitor, -NMMA, caused ~50% decrease in basal forearm blood low (from 3.04 ± 0.37 ml/100ml tissue/min to 1.49 ± 0.19 nl/100ml tissue/min; $p < 0.001$). Intrabrachial SNP (90 to 00 ng/min), an exogenous NO donor, was titrated to estore forearm blood flow back to baseline levels (2.77 ± .24 ml/100ml tissue/min; $p = 0.46$ clamp dose SNP versus aseline; paired Student *t* test). The inhibition of endoge-ous NO synthesis by the NO clamp attenuated SFLLRN-nduced vasodilation (Fig. 2B).

Potassium channel antagonism with TEA (1 mg/min) id not affect baseline blood flow ($p = 0.76$; data not hown). It attenuated vasodilation to SFLLRN (Fig. 2C), ut the combination of PGI_2, NO synthase, and potassium hannel inhibition appeared to abolish SFLLRN-induced asodilation (Fig. 2D).

Both with and without concurrent NO synthase and PGI_2 ynthase inhibition, TEA attenuated, but did not abolish rterial vasodilation to the control endothelium-dependent asodilator, bradykinin (Figs. 3A and 3B). Tetraethylammo-ium ion did not affect endothelium-independent forearm rterial vasodilation to SNP (Fig. 3C).

Endothelium-derived vasodilators and PAR-1–induced release of fibrinolytic and coagulant factors. The SFLLRN increased net t-PA antigen and activity and PAI-1 antigen release but did not affect net PAI-1 activity (Fig. 4). This increase was augmented by the NO clamp (Fig. 4) but was not affected by aspirin or TEA (data not shown; p = NS; ANOVA). Bradykinin caused a dose-dependent increase in net t-PA antigen ($p < 0.05$; ANOVA) and activity ($p < 0.0001$; ANOVA) release but did not affect PAI-1 antigen and activity release (p = NS for both; ANOVA). Tetraethylammonium ion did not alter bradykinin-induced PAI-1 or t-PA release (p = NS for both; ANOVA). Unpaired analysis between the 2 subject groups (protocol 4 vs. protocol 5) suggests that the NO clamp did not alter bradykinin-induced t-PA or PAI-1 release (p = NS for all; ANOVA). Neither bradykinin nor SFLLRN affected vWF release (data not shown; p = NS; ANOVA).

Endothelium-derived vasodilators and PAR-1–induced platelet activation. SFLLRN increased platelet-monocyte binding but this was unaffected by inhibition of NO or PGI_2 (Table 1). In contrast, SFLLRN increased beta-thromboglobulin ($p < 0.001$; ANOVA) that was augmented during the NO clamp ($p < 0.01$; ANOVA) (Fig. 5) but unaffected by aspirin (p = NS; ANOVA).

Discussion

Thrombin is one of the most powerful physiological agonists in the cardiovascular system, and its actions are fundamental to the processes of atherothrombosis. In a series of studies, we have here described the contrasting role of the endothelium in the PAR-1–mediated vascular actions of thrombin in vivo in man. Although not providing a major contribution to venoconstriction or PAI-1 release, the endothelium mediates PAR-1–induced arterial vasodilation and t-PA release. Our findings provide clear evidence of a major interaction between the vascular endothelium and thrombin in vivo in man. Furthermore, it highlights the critical importance of endothelial function at the time of acute arterial injury and intravascular thrombosis, such as during acute coronary syndromes.

Role of the endothelium in PAR-1–induced vasomotion. We have previously described the unexpected finding of PAR-1–induced venoconstriction in man (9). Although not caused by platelet aggregation (9), this effect could be mediated by either a direct action on vascular smooth muscle or via the release of endothelium-derived vasoconstrictors, such as endothelin or angiotensin II. To address this question, we assessed PAR-1 venoconstriction before and after endothelial denudation by instillation of distilled water. There was a modest trend toward enhanced venoconstriction after endothelial denudation, and we cannot exclude a small contribution from the endothelium that may also include the release of venodilatory mediators such as NO. However, PAR-1 continued to induce a marked dose-dependent venoconstriction even in the absence of the

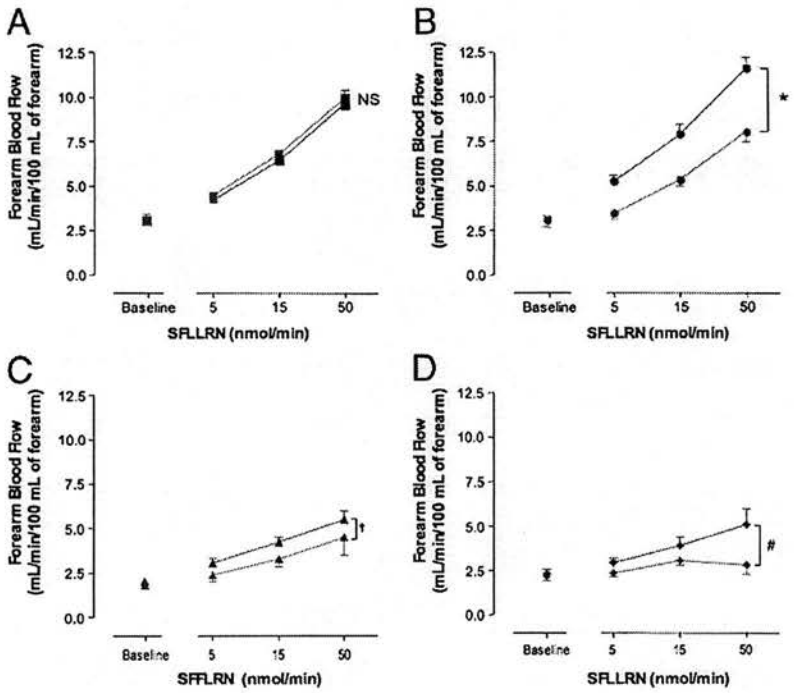

Figure 2 **SFLLRN-Induced Forearm Arterial Vasodilation**

In the presence **(red symbols)** and absence **(blue symbols)** of **(A)** aspirin **(squares)**, NS = nonsignificant ($p = 0.53$) in the presence versus the absence of aspirin (analysis of variance [ANOVA]); **(B)** the nitric oxide (NO) clamp **(circles)**, $*p < 0.0001$ in the presence versus the absence of the NO clamp (ANOVA); **(C)** tetraethylammonium (TEA) **(triangles)**, $\dagger p < 0.05$ in the presence versus the absence of TEA (ANOVA); and **(D)** aspirin, the NO clamp, and TEA **(diamonds)**, $\#p < 0.01$ in the presence versus the absence of aspirin, the NO clamp, and TEA (ANOVA).

ndothelium, which suggests a dominant and direct effect of PAR-1 on the vascular smooth muscle cells.

In contrast to effects on the venous circulation, the PAR-1 agonist causes potent arterial vasodilation. This uggests a different effect on the arterial vasculature that is ikely to be mediated by the endothelium. It would be ifficult and ethically challenging to conduct comparable in ivo endothelial denudation studies in the arterial circulaion of man. We chose, therefore, to use a pharmacological pproach to the inhibition of the 3 main known mediators of ndothelium-dependent vasodilation: PGI_2, NO, and EDHF. Although PGI_2 inhibition appeared to have no effect, inhibiion of NO and potassium channels both attenuated the PAR-1–induced vasodilation. Consistent with some cross talk nd compensatory up-regulation, combined inhibition of all asodilator mechanisms appeared to produce greater inhibiion, if not abolition, of the vasodilator actions of the PAR-1 gonist. This suggests that, unlike the venous circulation, PAR-1–mediated arterial actions are dominated by, and dependent on, the vascular endothelium.

PAR-1–induced release of endothelium-derived factors. n addition to vasomotion, PAR-1 has important effects on he release of endothelium-derived coagulant and fibrinoytic factors. In keeping with a wide range of other endohelial G-protein coupled receptor dilator agonists (19), we confirmed our earlier findings that the PAR-1 agonist causes endothelial t-PA release without affecting vWF. However, we also report here that SFLLRN-induced t-PA release appeared to be augmented by the inhibition of endogenous NO production. Smith et al. (20) have reported similar findings when they examined bradykinin-evoked t-PA release in the presence and absence of L-NMMA. Because t-PA release is independent of NO and cyclooxygenase activity, it has been suggested that EDHF is responsible for its release (21). One could speculate that, by inhibiting NO activity, EDHF is up-regulated, and this accounts for the augmented t-PA release induced by SFLLRN in our study and by bradykinin in Smith's study. However, in contrast to TEA's inhibitory effects on SFLLRN-induced arterial vasodilation, it had no effect on SFLLRN-evoked t-PA release. Similar findings have recently been reported by Muldowney et al. (22) who examined the role of EDHF in an in vitro model of thrombin-induced endothelial t-PA release. A variety of potassium channel antagonists, including TEA, had no effect on thrombin-induced t-PA release, but antagonists of specific epoxyeicosatrienoic acids appeared to inhibit thrombin-induced release of t-PA.

Another novel finding in our study was the increase in PAI-1 release, especially during NO synthase inhibition. To

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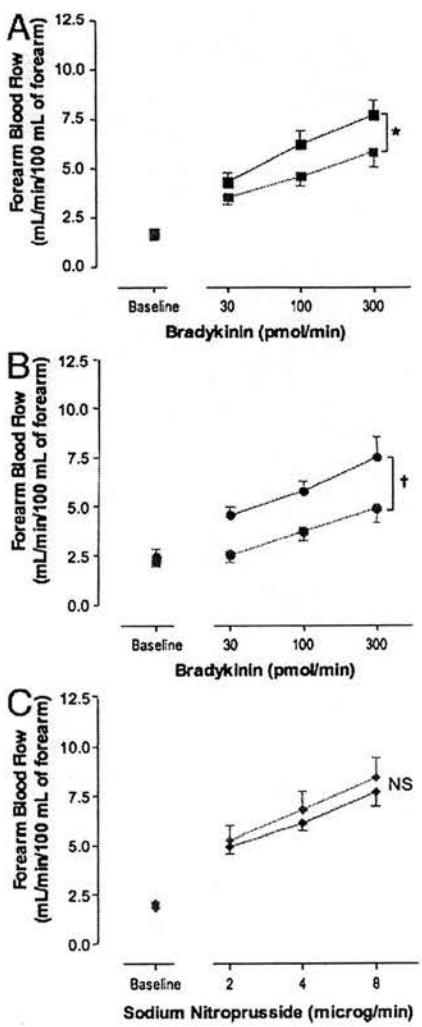

Figure 3 **Bradykinin- and SNP-Induced Forearm Arterial Vasodilation**

Forearm arterial vasodilation induced by **(A)** bradykinin **(squares)**, **(B)** bradykinin in the presence of the NO clamp and aspirin **(circles)**, and **(C)** sodium nitroprusside (SNP) **(diamonds)** in the presence **(red symbols)** and absence **(blue symbols)** of TEA. *p < 0.05; †p = 0.0001; NS = nonsignificant (p = 0.41) in the presence versus the absence of TEA (ANOVA). Abbreviations as in Figure 2.

late, there have been no reports of acute increases in plasma PAI-1 concentrations following the administration of endothelial agonists, especially using the forearm model (19). Even though the endothelium is an important source of PAI-1, we believe our findings are consistent with acute platelet release of PAI-1. There are several reasons to support our contention. First, although PAI-1 antigen concentrations increased, there was no corresponding rise in PAI-1 activity. Indeed, PAI-1 activity fell during marked release of t-PA. Plasminogen activator inhibitor type 1 is stored in platelet α-granules where its activity is very low (<5% of the activity seen in plasma) due to the absence of the stabilizing effect of vitronectin. In contrast, we would anticipate that endothelial-derived PAI-1 would remain active. Second, there was no concurrent rise in vWF confirming a selective effect on the endothelium with isolated t-PA release. Third, we also demonstrated concomitant platelet activation with marked increases in platelet-monocyte binding and release of beta-thromboglobulin; the latter is also stored in the α-granules of platelet. Finally, PAR-1–induced PAI-1 release was augmented during the NO clamp. Nitric oxide has important antiplatelet effects and, in the presence of its inhibition, increased platelet activation may have led to greater PAI-1 release.

Clinical relevance. Until recently, it has not been possible to undertake a safe clinical assessment of the vasomotor effects of thrombin due to its potent stimulatory effects upon the coagulation cascade. However, the synthetic activating peptide, SFLLRN, allows the examination of activation of the human PAR-1 thrombin receptor without activation of the coagulation cascade. This also permits the assessment of PAR-1 actions independent of the potential confounding effects that the activated coagulation pathway may have upon vascular responses.

We have demonstrated that many of the arterial effects of the PAR-1 agonist are dependent on, and mediated through, the endothelium and can therefore be used to assess endothelial function. To date, many endothelial G-protein-coupled receptor agonists have been used to assess endothelial function, such as acetylcholine and substance P. However, such agents are unlikely to have a major role in vascular physiology or pathophysiology and, as pharmacological tools, their relevance to the assessment of endothelial vasomotor function has limitations. As a more physiologically relevant tool, the PAR-1 agonist may be a more appropriate method of assessing endothelial function in the context of atherothrombosis. These novel insights into the vascular actions of the PAR-1 agonist will not only contribute to our understanding of human physiology and pathophysiology but also promise to inform the clinical development of novel antithrombotic PAR-1 receptor antagonists.

Study limitations. We chose to use SFLLRN as a PAR-1 agonist for several reasons. First, the vast majority of published work has employed SFLLRN as a PAR-1 activating peptide, and its actions have been widely characterized. Second, we have previous clinical experience of the in vivo actions of SFLLRN and this has facilitated the comparability of our current findings with our previous "first-into-man" clinical studies. Finally, SFLLRN is identical to the active cleaved sequence of the human PAR-1 receptor and represents a more physiologically relevant agonist of the receptor.

Although SFLLRN is selective for the PAR-1 receptor, it does have agonist activity at the PAR-2 receptor: 4-fold greater selectivity for the PAR-1 versus PAR-2 receptor (23). Therefore, there remains a possibility that a contribution of the observed actions of SFLLRN may represent PAR-2 antagonism. However, we do not believe this is likely for several reasons. First, we have

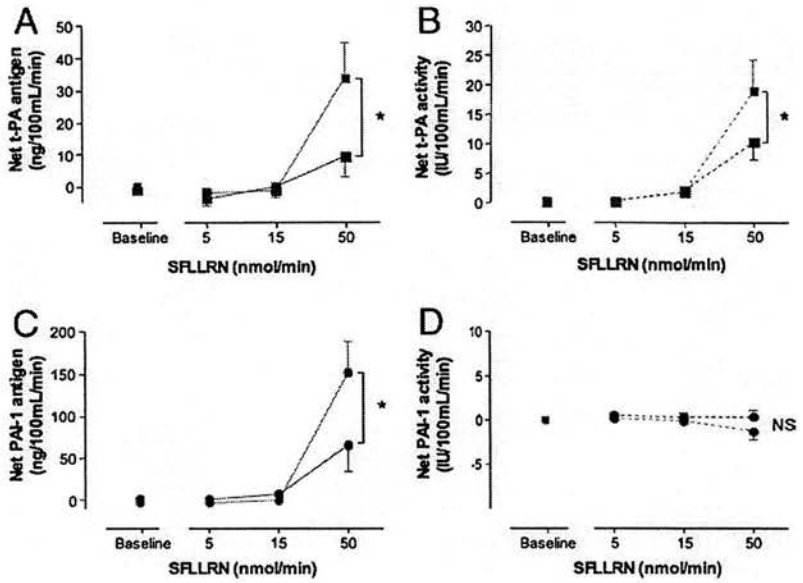

Figure 4 **SFLLRN-Induced t-PA and PAI-1 Release**

Net tissue-type plasminogen activator (t-PA) **(squares)** and plasminogen activator inhibitor type 1 (PAI-1) **(circles)** antigen **(solid lines)** and activity **(dashed lines)** in response to intrabrachial SFLLRN in the presence **(red symbols)** and absence **(blue symbols)** of the NO clamp. *$p < 0.0001$; NS = nonsignificant ($p = 0.075$) in the presence versus the absence of the NO clamp (ANOVA). Abbreviations as in Figure 2.

reviously shown that SLIGKV, a highly selective PAR-2 ctivating peptide, causes only modest arterial vasodilation t high doses and, in contrast to PAR-1 activation, causes narked venodilation and does not cause arterial t-PA elease in vivo (24). Moreover, the predicted end-organ oncentration of the highest dose of SFLLRN used in our urrent and previous studies is 4-fold lower than the median ffective dose for the PAR-2 receptor (23). However, we do ccept that, in future studies, consideration should be given o the use of the more selective PAR-1 activating peptide, FLLRN (23,25).

The role of NO in bradykinin-induced t-PA release emains controversial (19) and the present study has not efinitively addressed this issue. Although NO donors do ot induce t-PA release (26,27), inhibition of NO synthesis as been reported either to have no effect (21), or to increase 20), bradykinin-induced t-PA release. Our own unpaired nalysis of data from different subject populations is in eeping with the findings of Brown et al. (21) and suggests that bradykinin-induced t-PA release is unaffected by either NO or prostaglandin inhibition. Further research into the pathways involved in bradykinin-induced t-PA release is needed to clarify the role of NO and other potential mediators.

Conclusions

Protease-activated receptor type 1 activation causes contrasting effects in the human vasculature. It causes endothelium-dependent arterial vasodilation and t-PA release as well as endothelium-independent venoconstriction and PAI-1 release. There appears to be a major interaction between the vascular endothelium and thrombin's PAR-1–mediated effects in vivo in man. This highlights the critical importance of endothelial function particularly at the time of acute arterial injury and intravascular thrombosis, such as occurs during many acute cardiovascular events including myocardial infarction and stroke.

Table 1 **Percent Platelet-Monocyte Binding**

	Baseline		After SFLLRN	
	Infused Arm	Noninfused Arm	Infused Arm	Noninfused Arm
Placebo	16.62 ± 4.38	12.48 ± 1.61	75.89 ± 5.09*	41.36 ± 6.86*
Aspirin only	14.43 ± 1.95	14.87 ± 3.61	72.07 ± 5.41*	49.67 ± 7.82*
NO clamp only	11.84 ± 1.52	16.95 ± 3.23	81.21 ± 5.68*	46.42 ± 7.42*
Aspirin + NO clamp	12.19 ± 1.66	14.15 ± 2.02	82.85 ± 5.57*	46.63 ± 6.48*

*$p < 0.001$ versus baseline (analysis of variance).
NO = nitric oxide.

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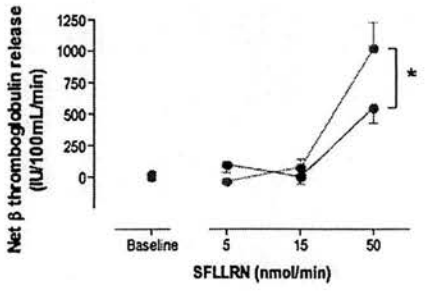

Figure 5 SFLLRN-Induced Beta-Thromboglobulin Release

In the presence **(red circles)** and absence **(blue circles)** of the NO clamp. *p < 0.01 net beta-thromboglobulin release induced by SFLLRN (50 nmol/min) in the presence versus the absence of the NO clamp (ANOVA). Abbreviations as in Figure 2.

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Marked Impairment of Protease-Activated Receptor Type 1-Mediated Vasodilation and Fibrinolysis in Cigarette Smokers

Smoking, Thrombin, and Vascular Responses In Vivo

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Objectives We sought to test the hypothesis that cigarette smoking adversely alters protease-activated receptor type 1 (PAR-1)-mediated vascular effects in vivo in humans.

Background Distinct from its role in the coagulation cascade, thrombin exerts its major cellular and cardiovascular actions via PAR-1. The activation of PAR-1 causes endothelium-dependent arterial vasodilation and the release of endogenous fibrinolytic factors.

Methods Forearm blood flow was measured with venous occlusion plethysmography in 12 cigarette smokers and 12 age- and gender-matched nonsmokers during intrabrachial infusions of PAR-1–activating-peptide (SFLLRN; 5 to 50 nmol/min), bradykinin (100 to 1,000 pmol/min), and sodium nitroprusside (2 to 8 μg/min). Plasma tissue plasminogen activator (t-PA) and plasminogen-activator inhibitor 1 antigen and activity concentrations were measured throughout the experiment.

Results All agonists caused dose-dependent increases in forearm blood flow ($p < 0.0001$ for all). Although bradykinin and sodium nitroprusside caused similar vasodilation, SFLLRN-induced vasodilation was attenuated in smokers ($p = 0.04$). Smokers had modest reductions in bradykinin-induced active t-PA release (reduced by 37%, $p = 0.03$) and had a marked impairment of SFLLRN-induced t-PA antigen ($p = 0.02$) and activity ($p = 0.006$) release, with a 96% reduction in overall net t-PA antigen release. The use of SFLLRN also caused similar (p = NS) increases in inactive plasminogen-activator inhibitor 1 in both smokers and nonsmokers ($p \leq 0.002$ for both).

Conclusions Cigarette smoking causes marked impairment of PAR-1–mediated endothelial vasomotor and fibrinolytic function. Relative arterial stasis and near abolition of t-PA release will strongly promote clot propagation and vessel occlusion. These findings suggest a major contribution of impaired endothelial PAR-1 action to the increased atherothrombotic risk of cigarette smokers. (J Am Coll Cardiol 2008;52:33–9) © 2008 by the American College of Cardiology Foundation

Smoking tobacco remains one of the most important and consistent modifiable risk factors for myocardial infarction and fatal coronary artery disease (1). The recent INTERHEART (A Study of Risk Factors for First Myocardial Infarction in 52 Countries and Over 27,000 Subjects) study revealed that smoking tobacco increases the risk of nonfatal myocardial infarction by as much as 7-fold (2). The pathophysiological mechanisms underlying this association are likely to be a combination of accelerated atherosclerosis (3) and a propensity to acute coronary thrombosis (1,4).

The endogenous fibrinolytic system is responsible for the dissolution of arterial thrombi that are frequently found on the surface of atherosclerotic plaques at areas of endothelial denudation (5,6). It is regulated by the profibrinolytic factor, tissue plasminogen activator (t-PA), and its endogenous inhibitor, plasminogen-activator inhibitor type 1 (PAI-1) (7–9). The rapid mobilization of t-PA from the endothelium is crucial, with thrombus dissolution being much more effective if t-PA is incorporated during, rather than after, thrombus formation (10). Indeed, acute stimu-

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Abbreviations and Acronyms

ANOVA = analysis of variance
EDHF = endothelium derived hyperpolarizing factor
NO = nitric oxide
PAI-1 = plasminogen activator inhibitor type 1
PAR = protease-activated receptor
t-PA = tissue-type plasminogen activator
vWF = von Willebrand factor

lated t-PA release predicts the future risk of cardiovascular events (11).

Thrombin plays a central role in the coagulation cascade and thrombosis. It is one of the most powerful physiological agonists in the cardiovascular system, and its actions are fundamental to the processes of atherothrombosis. Distinct from its enzymatic role in the coagulation cascade, thrombin causes direct cellular activation through stimulation of a novel family of G-protein-coupled receptors, protease-activated receptors (PARs) (12). These receptors have a unique mechanism of activation whereby agonist-induced proteolytic cleavage of the extracellular domain reveals a short peptide sequence that remains tethered and causes autoactivation of the receptor. To date, 4 different types of PARs have been identified: PAR-1, -3, and -4 are all activated by thrombin whereas PAR-2 is mainly activated by trypsin (13).

PAR-1 is the principal receptor that mediates the cardiovascular actions of thrombin. The hexapeptide, SFLLRN, represents the short peptide sequence revealed during PAR-1 activation and can be used as a selective agonist of the human PAR-1 thrombin receptor without activation of the coagulation cascade. Using SFLLRN, we have recently described the in vivo effects of PAR-1 activation in platelets, endothelium, and vascular smooth muscle in humans. For the first time, we were able to show that thrombin has unique and contrasting effects in the human vasculature, including arterial dilation, venous constriction, platelet activation, and tissue-type plasminogen activator (t-PA) release (14).

We, and others, have previously reported that pharmacological stimulation of acute t-PA release in the peripheral (15,16) and coronary (17,18) arterial circulations is markedly attenuated in smokers. In this study, we hypothesized that smokers have impaired PAR-1–mediated vascular responses. We, therefore, examined PAR-1–mediated t-PA release and vasomotor responses in the forearm circulation of cigarette smokers and healthy nonsmoking control subjects.

Methods

Subjects. Twelve healthy cigarette smokers (5 to 20 cigarettes/day) and 12 age- and gender-matched nonsmokers between ages 20 and 46 years participated in the study, which was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. The written informed consent of each subject was obtained before entry into the study. Exclusion criteria included a history of asthma, hypertension, diabetes mellitus, coagulopathy, hyperlipidemia, or vascular disease. Control subjects were lifelong nonsmokers and were not exposed to regular environmental tobacco smoke. Smokers had a history of regular daily cigarette smoking of at least 5 years and maintained their normal smoking habits in the week before attendance.

None of the subjects received vasoactive or nonsteroidal anti-inflammatory drugs in the week before the study, and all abstained from alcohol for 24 h before and from food, tobacco, and caffeine-containing drinks on the day of the study. All studies were performed in a quiet, temperature-controlled room maintained at 22°C to 24°C.

Intra-arterial drug administration. All subjects underwent brachial artery cannulation with a 27 standard-wire-gauge steel needle. The intra-arterial infusion rate was kept constant at 1 ml/min throughout all studies. Forearm blood flow was measured in the infused and noninfused arms by venous occlusion plethysmography with mercury-in-silastic strain gauges as described previously (15,19). Supine heart rate and blood pressure were monitored at intervals throughout each study with the use of a semiautomated noninvasive oscillometric sphygmomanometer.

After a 20-min intra-arterial infusion of 0.9% saline, the glycoprotein IIb/IIIa antagonist, tirofiban (1.25 μg/min; Merck, Sharp and Dohme, Hoddesdon, United Kingdom), was infused and continued throughout the study to inhibit potential PAR-1-induced platelet aggregation. This dose of tirofiban does not affect forearm blood flow (14).

During tirofiban administration, subjects received intra-arterial infusions of the PAR-1–activating peptide, SFLLRN (5, 15, and 50 nmol/min; Clinalfa, Läufelfingen, Switzerland), bradykinin (an endothelium-dependent vasodilator that causes the release of t-PA; 100, 300, and 1,000 pmol/min; Clinalfa), and sodium nitroprusside (an endothelium-independent vasodilator that does not release t-PA; 2, 4, and 8 μg/min; David Bull Laboratories, Warwick, United Kingdom). Study drugs were infused in random order for 10 min at each dose and were separated by a 20-min infusion of 0.9% saline.

Blood sampling. Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms. Blood samples were drawn simultaneously from each arm at the beginning of the study and during infusion of each dose of PAR-1–activating peptide (SFLLRN), bradykinin, and sodium nitroprusside. Venous blood was collected into acidified buffered citrate (Stabilyte, Trinity Biotech Plc, Co., Wicklow, Ireland; for t-PA assays) and into citrate (BD Vacutainer, BD UK Ltd., Oxford, United Kingdom; for PAI-1 and von Willebrand factor [vWF] assays). Samples were kept on ice before centrifugation at 2,000 *g* for 30 min at 4°C. Platelet-free plasma was decanted and stored at 80°C before assay. Plasma t-PA antigen and activity (t-PA Combi Actibind Elisa Kit, Technoclone, Vienna, Austria), PAI-1 antigen and activity (Elitest PAI-1 antigen and Zymutest PAI-1 Activity, Hy-

phen Biomed, Neuville-Sur-Oise, France), and vWF antigen (Dako A/S, Glostrup, Denmark) concentrations were determined with the use of enzyme-linked immunosorbent assays. Full blood count and hematocrit were measured at baseline and the end of the study.

Data analysis and statistics. Forearm plethysmographic data were analyzed as described previously (19). Estimated net release of plasma t-PA, PAI-1, and vWF has been defined previously as the product of the infused forearm plasma flow (based on the mean hematocrit and the infused forearm blood flow) and the concentration difference between the infused and noninfused arms (19). Variables are reported as mean ± SEM and analyzed with repeated measures analysis of variance (ANOVA) and a 2-tailed Student *t* test as appropriate. Statistical analysis was performed with GraphPad Prism (GraphPad Software, San Diego, California) and statistical significance taken at the 5% level. The authors had full access to the data and take responsibility for its integrity. All authors have read and agreed to the report as written.

Results

There were no differences in baseline characteristics between cigarette smokers and nonsmokers (Table 1). There were no changes in blood pressure, heart rate, or hematocrit (data not shown) during the study. Smokers had a mean cigarette consumption of 15 ± 1 cigarettes per day over a mean period of 9 ± 2 years (7 ± 2 pack-years).

Forearm blood flow. The use of tirofiban did not affect forearm blood flow (data not shown). Intra-arterial sodium nitroprusside, bradykinin, and the PAR-1–activating peptide, SFLLRN, all caused dose-dependent vasodilation in the infused arm of smokers and nonsmokers ($p < 0.0001$ for all; ANOVA). There were no changes in blood flow in the noninfused arm (data not shown).

Although there was no difference with bradykinin ($p = 0.64$; ANOVA smokers vs. nonsmokers), vasodilatation to SFLLRN was attenuated in smokers ($p = 0.044$; ANOVA smokers vs. nonsmokers). Endothelium-independent vasodilation evoked by the use of sodium nitroprusside was similar in both groups ($p = 0.74$; ANOVA smokers vs. nonsmokers) (Fig. 1).

Plasma fibrinolytic and hemostatic factors. Baseline plasma t-PA antigen and activity (Table 2) and vWF antigen (Table 3) concentrations were similar in smokers and nonsmokers. There appeared to be a trend toward greater absolute plasma PAI-1 antigen and activity concentrations in smokers, but this difference did not reach statistical significance (smokers vs. nonsmokers: PAI-1 antigen, $p = 0.07$ and $p = 0.10$, and PAI-1 activity, $p = 0.18$ and $p = 0.24$; infused and noninfused arms, respectively) (Table 4).

The use of SFLLRN caused a dose-dependent net release of t-PA antigen in nonsmokers ($p < 0.0005$; ANOVA) but not smokers ($p = 0.18$; ANOVA) (Fig. 2). In comparison with nonsmokers, the release of t-PA antigen and activity by SFLLRN was markedly attenuated in smokers ($p = 0.02$

Table 1 Baseline Subject Characteristics

	Nonsmokers	Smokers
Age, yrs	26 ± 2	29 ± 2
Gender, male/female	12/0	12/0
Body mass index, kg/m^2	24 ± 1	27 ± 1
Mean arterial pressure, mm Hg	97 ± 2	99 ± 2
Heart rate, beats/min	66 ± 2	64 ± 2
Baseline hematocrit	0.42 ± 0.01	0.42 ± 0.01

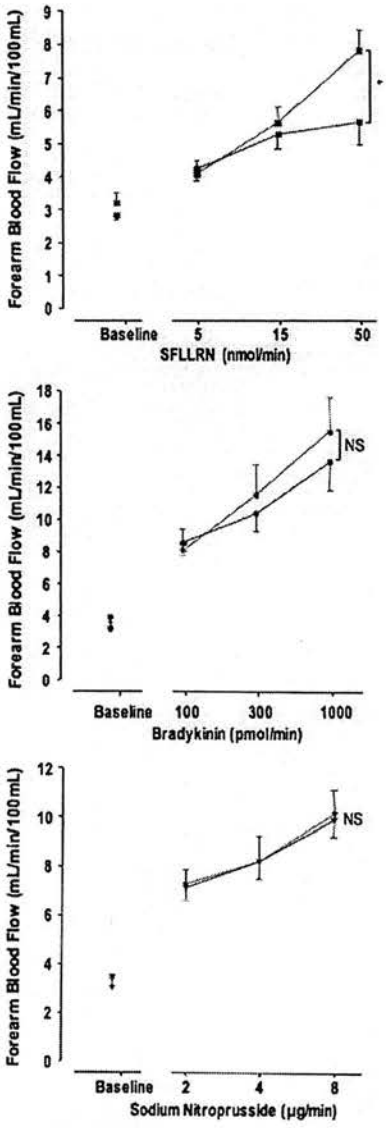

Figure 1 Forearm Arterial Vasodilation in Smokers and NonSmokers

Forearm arterial vasodilatation induced by SFLLRN **(squares, top panel)**, bradykinin **(circles, middle panel)**, and sodium nitroprusside **(triangles, bottom panel)** in smokers **(blue symbols)** and nonsmokers **(red symbols)**. *$p < 0.05$. NS = nonsignificant (analysis of variance, smokers vs. nonsmokers).

Table 2 Absolute Plasma t-PA Antigen and Activity Concentrations

	t-PA Antigen (ng/ml)				t-PA Activity (IU/ml)			
	Nonsmokers		Smokers		Nonsmokers		Smokers	
Arm	Infused	Noninfused	Infused	Noninfused	Infused	Noninfused	Infused	Noninfused
Baseline	8.15 ± 2.18	8.33 ± 2.24	10.83 ± 1.94	10.93 ± 2.23	0.48 ± 0.08	0.45 ± 0.06	0.36 ± 0.07	0.35 ± 0.07
Pre-SFLLRN	8.08 ± 2.07	8.41 ± 1.92	11.35 ± 2.2	11.15 ± 2.29	0.54 ± 0.08	0.44 ± 0.05	0.41 ± 0.07	0.40 ± 0.07
SFLLRN 5 nmol/min	7.84 ± 2.23	7.68 ± 1.76	9.91 ± 2.08	11.51 ± 2.70	0.63 ± 0.07	0.51 ± 0.05	0.44 ± 0.08	0.44 ± 0.08
SFLLRN 15 nmol/min	7.46 ± 2.08	7.74 ± 2.00	10.56 ± 1.85	10.83 ± 2.52	0.84 ± 0.11	0.59 ± 0.06	0.56 ± 0.11	0.50 ± 0.08
SFLLRN 50 nmol/min	11.54 ± 2.82*	6.93 ± 1.87	12.08 ± 2.58	11.17 ± 2.73	2.01 ± 0.38*	0.65 ± 0.09	0.78 ± 0.18†‡	0.49 ± 0.10
Pre-BK	7.14 ± 1.68	8.17 ± 2.43	10.66 ± 1.99	10.54 ± 2.12	0.55 ± 0.06	0.53 ± 0.07	0.35 ± 0.08	0.40 ± 0.08
BK 100 pmol/min	9.39 ± 1.76	7.55 ± 2.19	12.82 ± 2.76	10.44 ± 2.11	1.55 ± 0.21	0.54 ± 0.07	0.84 ± 0.15	0.45 ± 0.07
BK 300 pmol/min	9.32 ± 1.55	8.23 ± 2.42	13.66 ± 2.66	12.42 ± 2.78	2.15 ± 0.31	0.74 ± 0.10	1.60 ± 0.29	0.57 ± 0.12
BK 1000 pmol/min	11.55 ± 1.77*	8.44 ± 1.57	17.82 ± 3.49†	12.48 ± 2.70	3.51 ± 0.40*	0.97 ± 0.12	2.25 ± 0.45*‡	0.73 ± 0.13§

One-way analysis of variance for dose response: *$p < 0.005$, †$p < 0.01$; 2-way analysis: ‡$p < 0.001$, §$p < 0.05$ smokers versus nonsmokers.
BK = bradykinin; t-PA = tissue-type plasminogen activator.

and p = 0.006, respectively; ANOVA). However, SFLLRN induced a dose-dependent net release of PAI-1 antigen release in both nonsmokers (p = 0.0002; ANOVA) and smokers (p = 0.001; ANOVA). The response was similar in both groups (p = 0.36; ANOVA) and was associated with no change in net PAI-1 activity (p = NS for all; ANOVA [data not shown]) or vWF antigen release (p = NS; ANOVA) (Table 3).

Bradykinin caused a dose-dependent net release of t-PA antigen and activity in both smokers and nonsmokers ($p < 0.01$ for all; ANOVA). Bradykinin also evoked a dose-dependent increase in absolute t-PA activity in the noninfused arm of both nonsmokers ($p < 0.0001$; ANOVA) and smokers (p = 0.008; ANOVA). Net release of t-PA activity induced by bradykinin was less in smokers than nonsmokers (p = 0.032, smokers versus nonsmokers; ANOVA) (Fig. 2). Bradykinin caused no change in net PAI-1 antigen or activity and did not affect vWF antigen in either group (p = 0.91, nonsmokers; p = 0.98 nonsmokers; ANOVA). As expected (20). sodium nitroprusside caused no change in absolute or net release of t-PA, PAI-1 or vWf (data not shown).

Discussion

To our knowledge, we have shown for the first time that thrombin-mediated vascular responses are markedly impaired in cigarette smokers, with a substantial reduction observed in PAR-1–mediated endothelial t-PA release and forearm arterial vasodilation. This impaired vasomotor and fibrinolytic response may represent an important shift in the fine balance between intravascular thrombosis and fibrinolysis that could account for the increased incidence of atherothrombosis in cigarette smokers.

Smoking and PAR-1–induced arterial vasomotion. As reported by others (16), we observed no effect of smoking status on endothelium-dependent vasodilation to bradykinin or endothelium-independent vasodilation to sodium nitroprusside. One of the important novel observations from our study is that vasodilation evoked via PAR-1 is impaired in smokers, especially at the greater doses of SFLLRN. Because homeostatic mechanisms attempt to maintain vessel patency and minimize intravascular thrombus formation in healthy arteries, we have previously hypothesized the arterial vasodilation to PAR-1 activation represents a pro-

Table 3 Plasma von Willebrand Factor Concentrations During SFLLRN and Bradykinin Infusions

	von Willebrand Factor Antigen (ng/ml)			
	Nonsmokers		Smokers	
Arm	Infused	Noninfused	Infused	Noninfused
Baseline	0.72 ± 0.05	0.75 ± 0.19	0.77 ± 0.09	0.78 ± 0.13
Pre-SFLLRN	0.71 ± 0.06	0.80 ± 0.06	0.79 ± 0.07	0.71 ± 0.09
SFLLRN 5 nmol/min	0.70 ± 0.03	0.74 ± 0.05	0.72 ± 0.10	0.73 ± 0.06
SFLLRN 15 nmol/min	0.64 ± 0.03	0.73 ± 0.03	0.78 ± 0.06	0.63 ± 0.06
SFLLRN 50 nmol/min	0.70 ± 0.04	0.74 ± 0.04	0.78 ± 0.06	0.63 ± 0.06
Pre-BK	0.74 ± 0.06	0.74 ± 0.05	0.84 ± 0.10	0.74 ± 0.08
BK 100 pmol/min	0.76 ± 0.07	0.73 ± 0.05	0.78 ± 0.08	0.71 ± 0.05
BK 300 pmol/min	0.76 ± 0.06	0.74 ± 0.05	0.83 ± 0.08	0.68 ± 0.08
BK 1000 pmol/min	0.78 ± 0.06	0.76 ± 0.05	0.83 ± 0.08	0.76 ± 0.07

One-way analysis of variance for dose response, p = NS for all; 2-way analysis of variance, p = NS for all.
BK = bradykinin.

Table 4 Absolute Plasma PAI-1 Antigen and Activity Concentrations

	PAI-1 Antigen (ng/ml)				PAI-1 Activity (AU/ml)			
	Nonsmokers		Smokers		Nonsmokers		Smokers	
Arm	Infused	Noninfused	Infused	Noninfused	Infused	Noninfused	Infused	Noninfused
Baseline	23.58 ± 3.93	23.46 ± 3.71	40.09 ± 8.13	34.56 ± 5.50	0.80 ± 0.15	0.86 ± 0.15	1.48 ± 0.48	1.55 ± 0.60
Pre-SFLLRN	21.25 ± 3.35	21.68 ± 3.12	33.9 ± 7.19	33.08 ± 5.06	0.63 ± 0.12	0.73 ± 0.13	1.52 ± 0.56	1.57 ± 0.63
SFLLRN 5 nmol/min	21.03 ± 3.23	21.81 ± 3.36	38.52 ± 10.65	32.03 ± 6.04	0.62 ± 0.12	0.71 ± 0.14	1.52 ± 0.56	1.80 ± 0.61
SFLLRN 15 nmol/min	22.28 ± 3.55	21.13 ± 3.18	37.63 ± 9.06	32.92 ± 6.07	0.58 ± 0.12	0.72 ± 0.13	1.65 ± 0.53	1.63 ± 0.63
SFLLRN 50 nmol/min	31.56 ± 4.33*	20.52 ± 3.20	53.64 ± 10.20†	34.97 ± 5.86	0.55 ± 0.13	0.72 ± 0.14	1.59 ± 0.50	1.60 ± 0.67
Pre-BK	24.16 ± 3.86	21.58 ± 3.27	38.44 ± 9.67	37.41 ± 8.37	0.79 ± 0.17	0.81 ± 0.16	1.74 ± 0.57	1.70 ± 0.69
BK 100 pmol/min	24.83 ± 4.30	21.83 ± 3.13	38.13 ± 9.93	37.44 ± 7.90	0.66 ± 0.18	0.77 ± 0.17	1.54 ± 0.53	1.78 ± 0.75
BK 300 pmol/min	22.13 ± 3.24	23.68 ± 4.17	39.42 ± 0.39	37.10 ± 8.23	0.56 ± 0.16	0.76 ± 0.19	1.58 ± 0.56	1.35 ± 0.47
BK 1,000 pmol/min	20.57 ± 3.20	20.62 ± 2.91	34.53 ± 8.63	32.11 ± 6.57	0.45 ± 0.13	0.62 ± 0.16	1.33 ± 0.50	1.19 ± 0.40

One-way analysis of variance for dose response: *p < 0.05, †p < 0.0005; 2-way analysis of variance, p = NS for all, smokers versus nonsmokers.
BK = bradykinin; PAI-1 = plasminogen activator inhibitor type 1.

ective feedback mechanism. In the presence of a developing thrombus, PAR-1–mediated vasodilation will increase blood flow to limit arterial thrombosis by facilitating its rapid clearance and dissolution (14). Thus, this specific impairment of PAR-1–induced vasodilation may have major pathophysiological consequences during acute thrombotic events such as myocardial infarction.

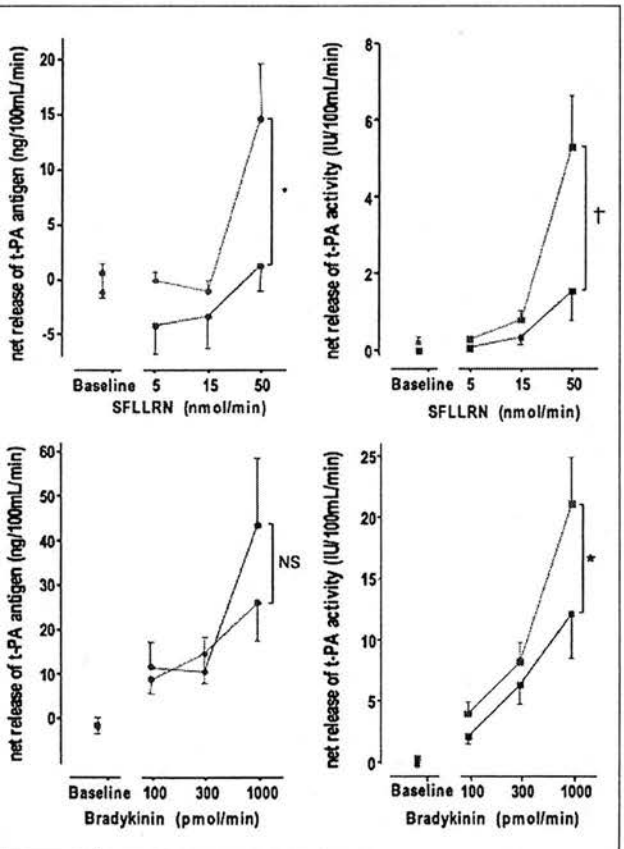

Figure 2 Net Release of t-PA Activator in Smokers and Nonsmokers

Net t-PA antigen **(circles)** and activity **(squares)** release induced by SFLLRN **(top panels)** and bradykinin **(bottom panels)** in smokers **(blue symbols)** and nonsmokers **(red symbols)**. *p < 0.05, †p = 0.005. NS = nonsignificant (analysis of variance, smokers vs. nonsmokers); t-PA = tissue-type plasminogen activator.

Smoking and PAR-1–induced release of endothelium-derived factors. Over and above diminished vasomotion, the major finding of our study was the almost complete abolition of PAR-1–mediated t-PA antigen release in cigarette smokers. Furthermore, PAR-1 activation caused only a very modest increase in t-PA activity despite causing substantial t-PA antigen and activity release in nonsmokers.

The current findings confirm previous studies from our own and other groups reporting reduced t-PA release in cigarette smokers (15,16,18). Although not demonstrated with t-PA antigen, the present finding of reduced bradykinin induced active t-PA release is consistent with similar observations previously reported by Pretorius et al. (16) However, the magnitude of the reduction in t-PA release is substantially greater for PAR-1–evoked responses than it is for bradykinin or substance P (96% vs. 40% to 50%) (15,16). We would therefore argue that SFLLRN-evoked t-PA release has the potential to be a more sensitive and pathophysiologically relevant assessment of endothelial vasomotor and fibrinolytic function.

Of note, PAR-1 activation also caused the release of PAI-1 antigen but did not cause an appreciable increase in PAI-1 activity, and neither indices were altered by smoking status. This increase in PAI-1 antigen without a change in activity suggests that SFLLRN is releasing PAI-1 from platelets rather than the endothelium because platelet-derived PAI-1 is relatively inactive as a result of the absence of the stabilizing effects of vitronectin (16,21). Furthermore, our own recent work has demonstrated a concomitant increase in beta-thromboglobulin, suggesting degranulation of platelet alpha granules (22). Therefore, the contribution of the endogenous fibrinolytic system to the prothrombotic state found in cigarette smokers is likely to be driven by impaired endothelial t-PA release and not by alterations in PAI-1 release or activity.

PAR-1 activation as a pathophysiologically relevant marker of endothelial function. The authors of previous studies to assess the endothelial release of endogenous fibrinolytic factors have used diverse methods. Historical means of stimulating t-PA release have included the systemic intravenous infusion of desmopressin and bradykinin, but this method causes significant confounding effects by altering systemic hemodynamics, activation of the sympathetic nervous system, and concomitant release of other mediators (6). By assessing the regional release of t-PA and PAI-1 in response to locally acting agonists, we can avoid such confounding effects.

We have previously demonstrated that substance P-induced t-PA release in the coronary (17) and peripheral (15) arterial circulations is impaired in cigarette smokers, and allows one to predict future adverse cardiovascular events in patients with coronary heart disease (11). However, although substance P has been a useful pharmacologic tool, it is unclear whether substance P is likely to act as a major pathophysiological mediator in atherothrombosis. In contrast, bradykinin may have a more direct role because it is released during the contact phase of coagulation and there is enhanced activation of the kallikrein system and bradykinin release in patients with acute coronary syndromes (23). However, we would argue that, given its central role in thrombosis and inflammation, thrombin is the most powerful and pathophysiologically relevant mediator in this setting. Our present findings not only reinforce previous findings but give the clearest indication yet that impaired endothelial function is of critical and dynamic importance in the setting of coronary heart disease and acute coronary syndromes.

Smoking and endothelium-dependent mechanisms. We have previously demonstrated that PAR-1 mediates arterial vasodilation via 2 endothelium-dependent mechanisms, namely nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) (22). The pathways via which PAR-1 activation causes the endothelial release of t-PA are less clear and, in fact, inhibition of NO synthesis causes augmented SFLLRN-induced t-PA release (22). This discrepancy has raised the question as to whether EDHF is responsible for t-PA release and, in the absence of NO, whether EDHF responses undergo a compensatory up-regulation. Although the bulk of evidence suggests that smoking predominantly affects endothelial function by increasing oxidative stress with consequent disruption of NO production (24,25), studies specifically examining the effect of smoking upon EDHF-mediated responses are lacking.

Study limitations. The forearm circulation has been an extremely reliable model for the assessment of vascular physiology and pathophysiology. We do accept that our findings in the forearm may not be accurately representative of the coronary circulation. However, we and others have previously demonstrated consistent findings of impaired endothelial t-PA release in both the forearm (15,16) and coronary (17,18) circulations of cigarette smokers. Although the forearm vascular bed is relatively protected from the development of atheroma, it therefore seems likely that changes in its fibrinolytic capacity are indicative of the coronary circulation.

Establishing the receptor-mediated effects of thrombin in the vasculature is of major physiological and therapeutic relevance. It could be argued that, in our studies, the safety requirement for the coadministration of tirofiban with SFLLRN detracts from these advantages. However, we used locally active doses of glycoprotein IIb/IIIa inhibitor that abolish SFLLRN-mediated platelet aggregation without affecting platelet-monocyte binding, a sensitive marker of platelet activation. Furthermore, it has no effect upon basal forearm blood flow or fibrinolytic responses to SFLLRN (14). We therefore believe that SFLLRN remains an important and relevant tool to assess these fundamental pathophysiological aspects of endothelial function.

We have demonstrated an important impairment of fibrinolytic capacity in smokers, but it remains unclear whether this reflects an impairment of synthesis, storage, and release of t-PA, or indeed acceleration of its degradation. Addressing these questions will be challenging and is likely to require specifically designed in vitro studies.

Conclusions

In healthy vessels, thrombin's powerful procoagulant and prothrombotic effects are offset by its ability to evoke the release of t-PA and induce arterial vasodilation. We have shown here that cigarette smoking causes a marked impairment in PAR-1–mediated endothelial vasomotor and fibrinolytic function. Relative arterial stasis and abolition of t-PA release will strongly enhance clot expansion and vessel occlusion. Taken together, these findings suggest a major contribution of impaired endothelial PAR-1 action to the increased atherothrombotic risk of smokers. These important and novel findings are of direct relevance to our understanding of the pathophysiology by which cigarette smoking causes an increased propensity to atherothrombotic disorders including acute myocardial infarction and stroke.

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Key Words: smoking ■ thrombosis ■ fibrinolysis ■ blood flow ■ endothelium.

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Sildenafil potentiates nitric oxide mediated inhibition of human platelet aggregation

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stract

Nitric oxide (NO) inhibits platelet aggregation primarily via a cyclic 3′5′-guanosine monophosphate (cGMP)-dependent process. denafil is a phosphodiesterase type 5 (PDE5) inhibitor that potentiates NO action by reducing cGMP breakdown. We hypothesised t sildenafil would augment the inhibitory effects of NO on in vitro platelet aggregation. After incubation with sildenafil or the soluble anylate cyclase inhibitor *H*-(1,2,4)oxadiazolo(4,3-*a*)quinoxallin-1-one (ODQ), collagen-mediated human platelet aggregation was essed in the presence of two NO donors, the cGMP-dependent sodium nitroprusside (SNP) and the cGMP-independent diethylamine zeniumdiolate (DEA/NO). SNP and DEA/NO caused a concentration-dependent inhibition of platelet aggregation. ODQ inhibited d sildenafil augmented the effect of SNP, and to a lesser extent the effect of DEA/NO. We conclude that sildenafil potentiates O-mediated inhibition of platelet aggregation through blockade of cGMP metabolism and that PDE5 inhibitors may have important iplatelet actions relevant to the prevention of cardiovascular disease.

words: Nitric oxide; cGMP; Phosphodiesterase inhibitors; Platelet aggregation

Nitric oxide (NO) is a potent vasodilator and inhibitor platelet aggregation. It is generated constitutively by ni- oxide synthase (NOS) in endothelial cells and platelets, it can be derived from an exogenous source such as NO nor drugs. The effects of NO are predominantly medi- d via stimulation of soluble guanylate cyclase (sGC) ich catalyses synthesis of the secondary messenger cyclic ′-guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) [1]. cGMP reduces platelet adhesion d aggregation via predominantly protein kinase C KC)-mediated pathways [2].

Phosphodiesterases are a group of enzymes responsible the breakdown of cyclic nucleotides including cyclic ′-adenosine monophosphate (cAMP) and cGMP. Phosphodiesterase type 5 (PDE5) is highly specific for cGMP metabolism, with inhibition of PDE5 leading to accumulation of cGMP and augmentation of the effects of NO. Sildenafil is a selective inhibitor of PDE5 and was originally developed as a potential antianginal drug because it augments the vasodilator effects of both endogenous NO, such as from the endothelium or platelets, and exogenous NO, such as from NO donor drugs. Although an ineffective antianginal therapy, it is an efficacious treatment for erectile dysfunction by augmenting the physical response to sexual stimulation and increasing penile blood flow by relaxing smooth muscle in the corpus cavernosum [3,4]. Recently, there has been renewed interest in the therapeutic potential of PDE5 inhibitors for other cardiovascular indications and small trials have shown benefit in the treatment of both primary [5–10] and secondary [11] pulmonary hypertension.

Given the importance of platelet activation in thrombosis associated with cardiovascular disease, it is important to

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determine whether sildenafil also affects platelet aggregation. Antiplatelet effects of sildenafil would have therapeutic implications for patients with cardiovascular disease both with respect to its potential use as a primary treatment and because patients requiring sildenafil for impotence frequently have other cardiovascular conditions. Previously published studies on the effects of sildenafil on platelet function have been inconclusive and contradictory [3,12,13].

We hypothesised that sildenafil would augment the inhibitory effects of NO donor drugs on collagen mediated human platelet aggregation in vitro and that the effects of sildenafil would be affected by the relative cGMP dependence of the NO donors. We therefore compared the effects of sildenafil on two different NO donors, sodium nitroprusside (SNP) and diethylamine diazeniumdiolate (DEA/NO), that inhibit platelet aggregation through cGMP-dependent and -independent mechanisms, respectively.

Materials and methods

Materials. Sildenafil (Pfizer, Sandwich, UK) was diluted in saline and kept at room temperature. ODQ (Tocris Cookson, Langford, UK) was dissolved in dimethyl sulphoxide (DMSO) prior to dilution in saline; final concentrations of DMSO did not exceed 0.5%. DEA/NO (Alexis, Nottingham, UK) was dissolved in 0.01 M NaOH. SNP (Sigma chemicals, USA) was dissolved in saline and protected from light. Collagen was obtained from Chrono-Log, Havertown, USA. All other reagents were obtained from Sigma, Poole, Dorset, UK.

Samples. Peripheral venous blood was drawn from healthy human non-smokers ($n = 25$) who had not taken any medications including anti-inflammatory drugs for at least 10 days. Blood was collected from the ante-cubital fossa using a 19 gauge butterfly needle into a 50 mL syringe and transferred into tubes containing 3.8% sodium citrate. The blood was centrifuged at 130*g* for 20 min at room temperature to obtain platelet rich plasma (PRP) and the platelet count was adjusted to levels between 200 and 250×10^9/L using autologous platelet poor plasma (PPP). Blood was centrifuged at 1200*g* for 10 min to obtain PPP for this purpose and for reference samples.

Platelet aggregation. Platelet aggregation was measured using standard optical platelet aggregometry [14]. PRP samples were equilibrated at 37 °C and stirred continuously. Platelets were incubated with sildenafil, 1 μM [3,12], for 5 min, or the selective sGC inhibitor ODQ, 20 μM [15], for 5 min. PRP was then treated with the NO-donors SNP or DEA/NO (0.1–10 μM; 1 min), before induction of platelet aggregation with a supramaximal concentration of collagen (2.5 μg/mL). Aggregation was monitored for 5 min in a four channel aggregometer (Chronolog 470 VS, Labmedics, Stockport, UK) linked to a MacLab 4s analogue-digital converter (AD Instruments, Sussex, UK) and Chart software (AD instruments, Sussex, UK). All results are expressed as a percentage of the maximal collagen-induced aggregation.

Statistical analysis. Data were analysed using GraphPad Prism software and results are expressed as means ± SEM. Statistical significance was taken at 5% level. Two-way analysis of variance (ANOVA) was used to assess differences between the groups.

Results

Addition of both NO-donor drugs, SNP and DEA/NO, at concentrations of 0.1–10 μM caused concentration-dependent inhibition of collagen (2.5 μg/mL) mediated platelet aggregation ($n = 25$; ANOVA, $p < 0.0001$ for both SNP and DEA/NO; Fig. 1).

cGMP-dependence of SNP and DEA/NO

The inhibitory effects of SNP (0.1–10 μM) on collagen mediated platelet aggregation were almost abolished by 15 min incubation with ODQ (20 μM) ($n = 11$; ANOVA, $p < 0.001$; Fig. 1A). ODQ also reduced the inhibitory effects of DEA/NO ($n = 12$, $IC_{50} = 4.7 \times 10^{-9}$ M versus 8.2×10^{-7} M, $p < 0.001$; Fig. 1B) but to an apparently lesser degree than those of SNP.

Effects of sildenafil on NO mediated inhibition of platelet aggregation

Pre-incubation with sildenafil (1 μM) for 5 min augmented the inhibitory effects of both SNP ($IC_{50} = 2.6 \times 10^{-6}$ M versus 3.6×10^{-7} M; $n = 14$; ANOVA, $p < 0.0001$; Fig. 2A) and to a lesser extent than those of DEA/NO ($IC_{50} = 1.6 \times 10^{-7}$ M versus 1×10^{-7} M; $n = 13$; ANOVA, $p < 0.0001$; Fig. 2B). Sildenafil (1 μM) had no effects on its own (62 ± 3% versus 62 ± 4%; $n = 17$, $p =$ ns, paired t test) on collagen mediated platelet aggregation.

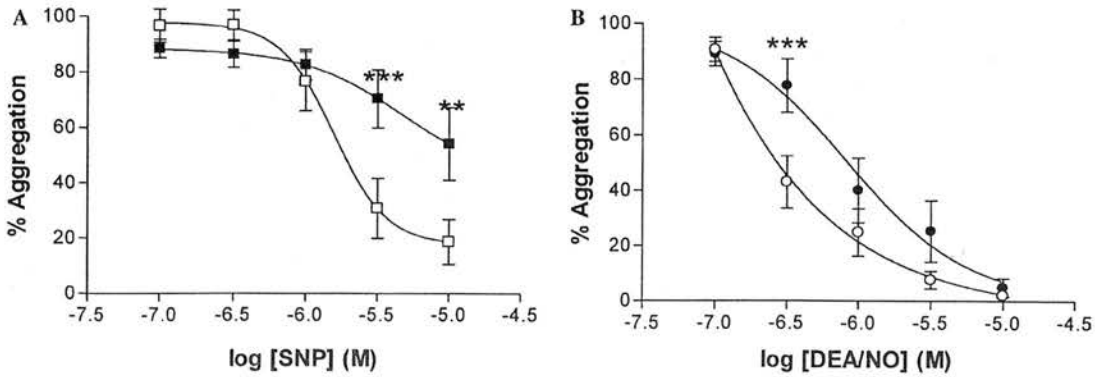
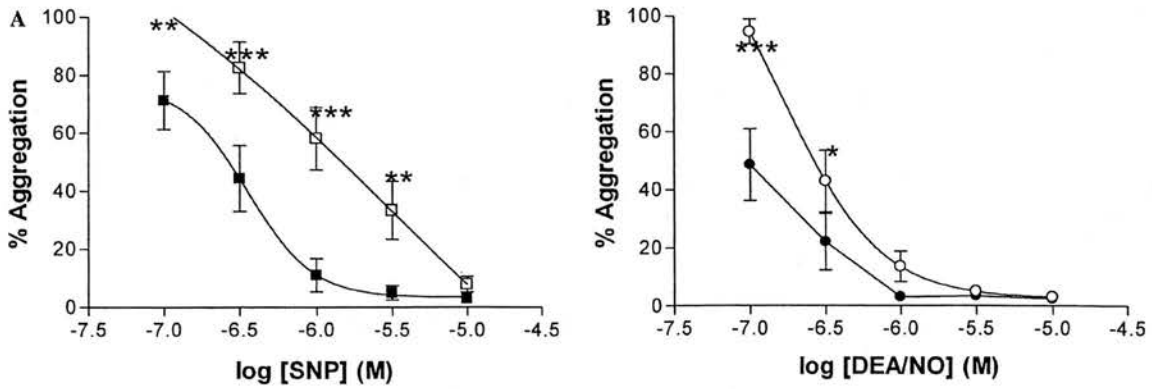


Fig. 1. (A,B) Inhibition of collagen induced platelet aggregation by sodium nitroprusside (SNP; squares) and diethylamine diazeniumdiolate (DEA/NO; circles) in the presence (closed symbols) or absence (open symbols) of *H*-(1,2,4)oxadiazolo(4,3-*a*)quinoxallin-1-one (ODQ). $^{***}p < 0.001$, $^{**}p < 0.01$.



2. (A,B) Inhibition of collagen induced platelet aggregation by sodium nitroprusside (SNP; squares) and diethylamine diazeniumdiolate (DEA/NO; les) in the presence (closed symbols) or absence (open symbols) of sildenafil. $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$.

cussion

We have shown that sildenafil alone does not inhibit telet aggregation but has a powerful potentiating ef- t on the antiplatelet effects of NO donor drugs. In ping with the relative cGMP-dependence of the two donor drugs tested, SNP appeared to be more sensi- e to sildenafil-mediated augmentation. We conclude t sildenafil predominantly potentiates cGMP-depen- nt NO mediated inhibition of platelet aggregation. is may have relevance to the potential cardiovascular cts of sildenafil.

We selected two different NO donors for our studies, P and DEA/NO. SNP is a cGMP-dependent NO nor that acts by diffusing into the platelet where it dergoes bioactivation to release NO and activate C, leading to the production of cGMP. In contrast, EA/NO is hydrolysed spontaneously at physiological mperature and pH, and releases NO primarily in the racellular compartment, resulting in antiplatelet ef- ts that are largely cGMP-independent [14,15]. Sepa- e experiments with similar diazeniumdiolates indicate t these effects are mediated by increased sequestra- n of Ca^{2+} into the sarcoplasmic reticulum through ivation of the sarco-endoplasmic reticulum Ca^{2+} Pase (SERCA) [16]. In keeping with previous studies], the sGC inhibitor ODQ nearly completely inhibited effects of SNP, suggesting cGMP dependence, whilst nly modestly affected DEA/NO. Furthermore, silde- il was found to potentiate the effects of both SNP l DEA/NO, but this effect was most marked with P.

Previously published studies have been inconclusive l inconsistent. Some have suggested that sildenafil ght augment thrombin induced platelet aggregation], whilst others describe an independent inhibitory ef- of sildenafil [17]. Consistent with our own findings, llis et al. [3] reported that sildenafil does not have di- t effects, but does augment the inhibitory effects of an donor. The lack of effect of sildenafil alone on collagen-induced platelet aggregation is perhaps surprising, given that platelets are known to contain constitutive nitric oxide synthase and to generate sufficient NO to modulate aggregatory responses [18,19]. It is, however, worth noting that activation of constitutive nitric oxide synthases is highly dependent on intracellular Ca^{2+} that, in the case of platelets, is only elevated during activation. Faced with a strong stimulus in the form of collagen, it is likely that any increase in cGMP levels will be "too little, too late" to have any impact on the aggregation process. It does not, however, rule out an in vivo effect of sildenafil on the activation process. Platelets are continuously exposed to endothelium-derived NO as well as that from platelets themselves, and at least some of the activation stimuli are less intensive than the in vitro exposure to collagen.

We conclude that PDE 5 inhibitors may have important antiplatelet effects that have potential in the treatment of cardiovascular diseases.

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