THROMBOTIC AND ENDOTHELIAL FACTORS IN THE CONTROL OF HUMAN VASCULAR FUNCTION

BY

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ABSTRACT

Background: The endothelium is crucial for the control of vascular function and for the maintenance of vascular health. In addition to nitric oxide (NO) and prostacyclin (PGI_2), endothelium-derived hyperpolarising factor (EDHF) is a third endothelium-derived vasodilator. It has also been implicated with a role in the release of the endogenous fibrinolytic factor, tissue plasminogen activating factor (t-PA). Potassium channels appear to be vital for its mechanism and gap junctions have been implicated with a role, but the identity of EDHF remains elusive. Consequently, it remains unmanipulated as a therapeutic target despite its involvement in a wide variety of vascular diseases. Additionally, it has not previously been possible to examine safely the *in vivo* interaction between thrombotic factors and endothelium-derived mediators, including EDHF. Protease-activated receptor type 1 (PAR-1) is the principal thrombin receptor in man. By examining the effects of direct PAR-1 receptor activation, it is possible to examine the *in vivo* vascular effects of thrombin in isolation from its stimulatory effects upon the coagulation cascade in both health and disease.

Objectives: 1) To assess the role of gap junctions and their connexin components in EDHF-mediated vasorelaxation in human resistance arteries *in vitro*. 2) To evaluate the vascular effects of potentiation of communication via the connexin 43 gap junction subunit *in vivo*. 3) To evaluate the role of the endothelium and endothelium-derived factors in the mediation of PAR-1 evoked vasomotion and t-PA release *in vivo*. 4) To examine the effect of cigarette smoking upon endothelial PAR-1 responses *in vivo*.

Methods: Fat biopsies were obtained from women undergoing elective caesarean section. Subcutaneous resistance arteries were subsequently dissected out. Vascular responses were assessed in a wire-myograph and the role of connexins assessed using specific connexin antagonists, connexin mimetic peptides (CMPs). Dorsal hand vein tone was assessed using the Aellig technique. Responses to local intravenous infusion of SFLLRN, a synthetic PAR-1 activating peptide, were assessed before and after venous endothelial denudation achieved by the local instillation of distilled water. Forearm venous occlusion plethysmography, with venous blood sampling, was used for the assessment of peripheral arterial blood flow and endogenous fibrinolytic responses *in vivo*. Intra-brachial rotigaptide, a novel peptidic agent, was used to assess the vascular effects of augmentation of intercellular communuication via connexin 43. Intra-brachial SFLLRN was infused in the presence and absence of aspirin, the 'nitric oxide clamp' and tetraethylammonium ion (TEA, a calcium-activated potassium channel antagonist) to assess the contribution of PGI_2, NO and EDHF respectively, in the mediation of the vascular effects of PAR-1 activation. The vascular effects of intra-brachial SFLLRN were compared in smokers and non-smoking matched control volunteers.

Results: EDHF-mediated vasorelaxation was abolished by the combined antagonism of the three major vascular connexins *in vitro*. Inhibition of connexin 43 alone reproduced this response but antagonism of the other vascular connexins alone was without effect, despite immunohistochemical evidence of their expression. However,

potentiation of communication via connexin 43 by intra-arterial rotigaptide did not affect endothelium-dependent vasodilatation or t-PA release *in vivo*. PAR-1 activation caused venoconstriction in the presence and absence of endothelium. PAR-1 mediated peripheral arterial vasodilatation was not affected by aspirin but was attenuated by the 'nitric oxide clamp' and by TEA alone, and was almost abolished by their combination. PAR-1 activation caused t-PA release that was not affected by aspirin, the 'nitric oxide clamp' or TEA. PAR-1 evoked t-PA antigen release was almost completely absent in cigarette smokers despite marked release in non-smokers. Cigarette smokers displayed attenuated PAR-1 mediated peripheral arterial vasodilatation.

Conclusions: Connexin 43 is vital for the mediation of EDHF activity in pregnant human resistance vessels *in vitro*. However, augmentation of connexin 43 communication has no effect on arterial vasodilatation or t-PA release in the peripheral circulation of healthy man. It remains to be established whether augmentation of connexin 43 communication improves endothelial function in patients with vascular disease. Acting via PAR-1, thrombin makes a major interaction with arterial, but not venous, endothelium to cause vasodilatation via NO- and EDHF-dependent pathways. It causes the endothelial release of t-PA that is independent of PGI_2, NO and EDHF. The arterial effects of PAR-1 activation are markedly impaired in association with endothelial dysfunction caused by smoking cigarettes. Relative arterial stasis and near abolition of t-PA release will strongly promote clot propagation and vessel occlusion. These findings highlight the important interaction between thrombotic and endothelial factors at times of acute arterial injury such as occurs during acute myocardial infarction and stroke.

To my wife, Stephanie

CONTENTS

DECLARATION

This thesis has been composed entirely by me and has not been submitted for any other degree or professional qualification. The presented work has been undertaken by me, except for the electrophysiological mapping studies presented in Chapter 4 which were conducted by Dr. Rachel Myles at Glasgow University. The dorsal hand vein studies described in Chapter 4 were performed by Dr. Ingibjorg Guðmundsdóttir who also performed the initial 'nitric oxide clamp' forearm arterial studies in eight of the volunteers in that chapter. Dr. Guðmundsdóttir performed ten of the forearm arterial studies in Chapter 6.

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CHAPTER 1

INTRODUCTION

1.1 ENDOTHELIAL FUNCTION

The vascular endothelium forms a single cell layer that covers the luminal surface of all blood vessels under normal conditions. Despite knowledge of its existence since the mid 1800s [Schwann, 1847; Hwa and Aird, 2007], its vital physiologic roles were not recognised until more than a century later. Prior to the seminal work of Furchgott and Zawadzki [Furchgott and Zawadzki, 1980], there had been little appreciation that the endothelium was anything more than a passive, semi-permeable barrier between intraluminal blood and vascular smooth muscle or the interstitial space. However, our understanding of the critical roles it fulfils has now deepened. Not only is the endothelium intimately involved with the regulation of vasomotion [Furchgott and Zawadzki, 1980], it also plays a pivotal role in coagulation and fibrinolytic processes [Emeis, 1992]. It is responsible for mounting a response to vascular injury, exerts important anti-inflammatory activities and has powerful influence upon the migration of vascular smooth muscle cells and the adhesion and aggregation of platelets [Cines *et al*, 1998; Félétou and Vanhoutte, 2006; Rao *et al*, 2007]. These homeostatic properties are mediated by a variety of factors synthesised and released by the endothelium in response to mechanical, neural and humoral stimuli.

1.2 ENDOTHELIUM-DERIVED FACTORS AND THE CONTROL OF VASOMOTION

Endothelial stimulation results in the immediate release of a number of vasodilator mediators including prostacyclin (PGI_2), nitric oxide (NO) and endothelium-derived

hyperpolarising factor (EDHF). Moreover, the endothelium is also able to generate and release vasoconstrictor mediators, such as angiotensin II and endothelin-1, and hence provide a counter-regulatory tonic action on the adjacent vascular smooth muscle [Haynes *et al*, 1996].

1.2.1 NITRIC OXIDE

Nitric oxide was the first endothelium-derived vasodilator to be described [Furchgott and Zawadzki, 1980] and is now well characterised. In addition to its potent vasodilator effects, NO exerts many other vascular roles including the inhibition of vascular smooth muscle proliferation and the regulation of interactions between leucocytes and the blood vessel wall. Intravascular NO is principally taken up by haemoglobin but also enters platelets to cause inhibition of their activation, adhesion and aggregation [Moncada and Higgs, 2006].

Nitric oxide is produced during the oxidation of L-arginine to L-citrulline [Palmer *et al*, 1988] under the enzymatic control of NO synthase. Endothelial nitric oxide synthase (eNOS) is constitutively expressed and NO is responsible for the tonic relaxation of blood vessels in the systemic and pulmonary circulations at rest [Vallance *et al*, 1989; Haynes *et al*, 1993; Stamler *et al*, 1994]. Endothelial nitric oxide synthase may also be activated via a calcium-dependent mechanism and, in addition to its role in the maintenance of basal vascular tone, NO is released from the endothelium in response to a number of agonists and by shear stress. In the human arterial circulation *in vivo*, agonists including bradykinin, substance P and acetylcholine evoke vasodilatation by endothelium-dependent mechanisms including

the stimulation of endothelial NO synthesis and release [Rongen *et al*, 1993; Cockcroft *et al*, 1994] (Table 1.1).

Table 1.1 Endothelium-dependent vasodilator agonists

Acetylcholine	PAR-1 activating peptide (SFLLRN)
Bradykinin	Thrombin
Substance P	Adenine triphosphate (ATP)
Histamine	Uridine triphosphate (UTP)
Methacholine	Serotonin (5-HT)

PAR-1 - protease-activated receptor type 1.

Once synthesised, the pathway via which NO acts to evoke vasodilatation is well understood. Following its release from the abluminal aspect of the endothelium, it activates smooth muscle guanylate cyclase and increases intracellular guanosine monophosphate [Arnold *et al*, 1977]. The resultant decrease in the smooth muscle intracellular concentration of calcium causes vascular relaxation [Collins *et al*, 1986]. Exogenous NO donors, such as sodium nitroprusside and nitroglycerin, also act via this mechanism [Kukovetz *et al*, 1979] but bypass the requirement for a functional endothelium.

Constitutive NO synthase can be inhibited *in vitro* and *in vivo* with guanidine-substituted analogues of L-arginine. These analogues inhibit NO synthase by competition with L-arginine for the substrate binding site and include L-N^G-monomethyl arginine (L-NMMA) and its methyl ester, N^{ω}-nitro-L-arginine-methyl ester (L-NAME).

1.2.2 PROSTACYCLIN

In addition to the production of NO, the endothelium is capable of the synthesis of vasoactive prostanoids, including PGI_2. As well as causing endothelium-dependent vasodilatation, PGI_2 exerts important antithrombotic and anti-platelet activity [Moncada, 1982]. It is produced from arachidonic acid via cyclooxygenase and is rapidly synthesised and released in the endothelium in response to agonists including bradykinin and adenosine nucleotides. Signal transduction involves the activation of adenylate cyclase with consequent elevation of intracellular cyclic adenosine monophosphate (cAMP) [Moncada et al, 1977a].

The vasodilating effects of PGI_2 are less marked than those of NO in the majority of vascular beds. However, it does contribute to resting vascular tone in the human forearm although the reported magnitude of its contribution has varied considerably between studies [Duffy *et al*, 1998; Campia *et al*, 2002; Singh *et al*, 2002]. Endothelium-dependent vasodilating agonists have been shown to act, in part, via PGI_2 *in vitro* [Toda, 1977] and *in vivo* in animals [Türker *et al*, 1982; Copeland *et al*, 1995]. However, this finding has been less consistent *in vivo* in man [Benjamin *et al*, 1989; Davie and McMurray, 2002].

The synthesis of PGI_2 can be blocked with inhibitors of cyclooxygenase such as aspirin [Heavey *et al*, 1985] and indomethacin [Moncada *et al*, 1977b].

1.2.3 ENDOTHELIUM-DERIVED HYPERPOLARISING FACTOR

In addition to NO and PGI_2, evidence points to the existence of a third powerful vasodilator called EDHF. Although the roles and mechanisms of NO and PGI_2 are well characterised, EDHF remains poorly understood.

After pharmacologic blockade of both NO and PGI_2, a substantial degree of endothelium-dependent vasodilatation is still observed and is associated with smooth muscle hyperpolarisation. This activity is attributed to EDHF [Vanhoutte, 1987; Bény and Brunet, 1988; Chen *et al*, 1988; Feletou and Vanhoutte, 1988; Chen *et al*, 1991; Mombouli *et al*, 1996; Feletou and Vanhoutte, 1999; Vanhoutte, 2004]. Furthermore, murine gene knockout models engineered to lack both cyclooxygenase and eNOS also retain the capacity for endothelium-dependent vasodilatation [Scotland *et al*, 2005].

Physiologic Role of EDHF

In humans, EDHF-mediated vasodilatation has been described in various vascular beds including coronary [Nakashima *et al*, 1993; Kemp and Cocks, 1997; Miura *et al*, 2003], internal mammary [Liu *et al*, 2000; Deja *et al*, 2005; Wei *et al*, 2007], radial [Hamilton *et al*, 2001; Wei *et al*, 2007], renal [Kessler *et al*, 1996], mesenteric [Mackenzie *et al*, 2008], subcutaneous [Luksha *et al*, 2004; Mackenzie *et al*, 2008] and cerebral arteries [Petersson *et al*, 1997]. Consistently, EDHF's role as a vasodilator is most prominent in the smaller resistance arteries [Shimokawa et al, 1996; Urakami-Harasawa et al, 1997; Tomioka et al, 1999; Berman et al, 2002] that are responsible for the control of systemic blood pressure and local tissue perfusion.

Studies examining the role of EDHF in the maintenance of resting forearm arterial tone have yielded inconsistent findings [Honing et al, 2000; Inokuchi et al, 2003] and *in vivo* assessments of the role of EDHF in the control of systemic blood pressure in man are lacking. Endothelium-derived hyperpolarising factor serves as an important back-up mechanism when NO bioavailability is impaired and alterations in its activity have commonly been noted in association with a wide variety of disease states [Feletou and Vanhoutte, 2004].

Generation of Endothelial Hyperpolarisation

Endothelium-derived hyperpolarising factor has been defined as a dilator process which: (i) requires an intact endothelium; (ii) is distinct from both endothelium-derived NO or PGI_2; (iii) dilates by hyperpolarising vascular smooth muscle; and (iv) involves calcium-activated potassium channels (K_{Ca}) [Golding et al, 2002] (Figure 1.1).

The mechanism via which endothelial cells generate hyperpolarisation in response to agonists is reasonably well understood. Agonists evoke the release of calcium ions (Ca^{2+}) from intracellular stores via the activation of phospholipase C and inositol triphosphate-gated calcium release channels. This initial increase in the intracellular concentration of Ca^{2+} elevates the transmembrane electrochemical gradient such that influx of Ca^{2+} into the endothelium from the extracellular space is sustained to allow the activation of endothelial small, intermediate and occasionally large conductance calcium-activated potassium channels (SK_{Ca}, IK_{Ca} and BK_{Ca} respectively.) Potassium efflux via these channels is a prerequisite for endothelial hyperpolarisation

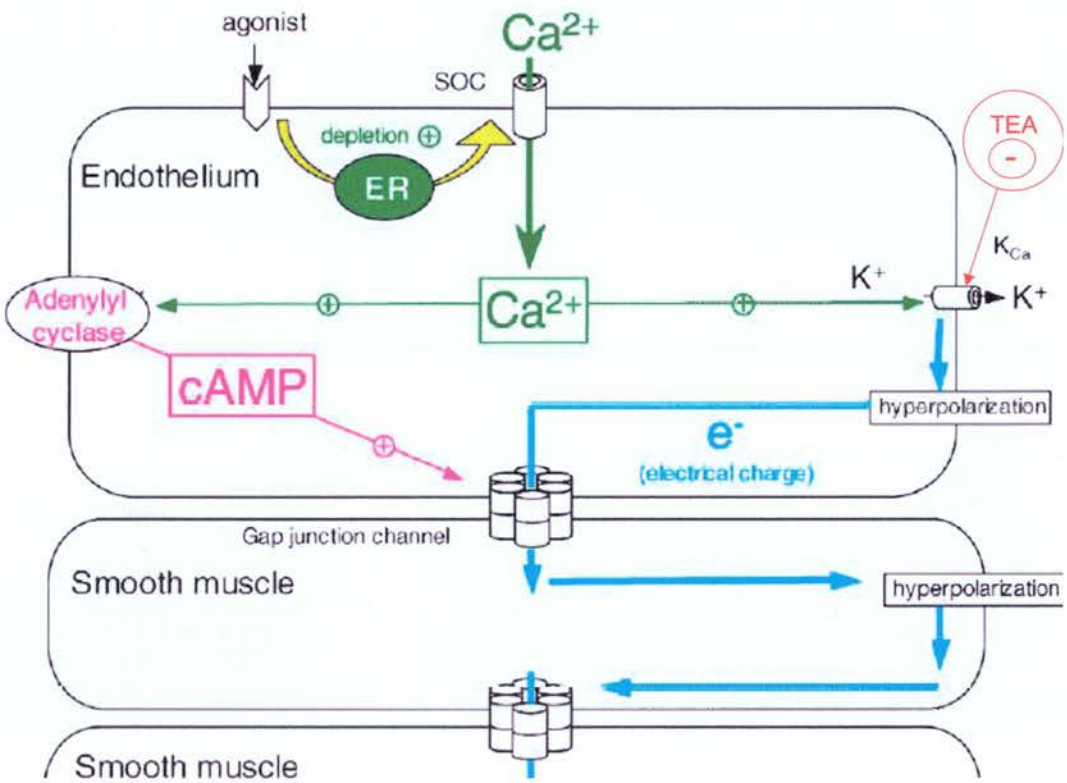


Figure 1.1 *The endothelial generation of hyperpolarisation. The change in membrane potential that initiates relaxation follows the opening of calcium (Ca^{2+}) –activated K^+ channels (K_{Ca}) by agonist-induced elevations in cytosolic free Ca^{2+} entry (CCE) via store-operated channels (SOC) triggered by depletion of the endoplasmic reticulum (ER) Ca^{2+} store. Endothelial hyperpolarisation is conducted to subjacent smooth muscle cells via myoendothelial gap junctions. Elevations in cytosolic Ca^{2+} may also elevate cAMP levels by stimulating adenylyl cyclase, thereby enhancing the electrical conductance of myoendothelial gap junctions (Section 1.3). Tetraethylammonium ion (TEA) exerts antagonistic actions at K_{Ca} [Griffith et al, 2004].*

as demonstrated by several lines of investigation: (i) studies employing radiolabelled rubidium to track the passage of potassium ions (K^+) demonstrates their efflux in association with hyperpolarisation of suitably loaded endothelial cells [Gordon and Martin, 1983]; (ii) there is an inverse relationship between the amplitude of agonist-induced changes in endothelial membrane potential and the prevailing concentration of extracellular K^+ [Mehrke and Daut, 1990]; and (iii) hyperpolarisation is either attenuated or abolished in the presence of peptidic inhibitors of K_{Ca} [Edwards *et al*, 1998; Frieden *et al*, 1999; Ohashi *et al*, 1999; Griffith, 2004] and in a murine model engineered to lack endothelial IK_{Ca} [Si *et al*, 2006]. Importantly, SK_{Ca}, IK_{Ca} and BK_{Ca} have all been isolated in endothelial cells [Nilius and Droogmans, 2001].

K_{Ca} Antagonists

Endothelium-dependent hyperpolarisation and EDHF-mediated vasodilatation is inhibited *in vitro* by a variety of K_{Ca} antagonists, but most consistently by the dual application of apamin (SK_{Ca} antagonist) and charybdotoxin (non-selective IK_{Ca} and BK_{Ca} antagonist, with additional effects at voltage-dependent potassium channels) [Edwards *et al*, 1998; Frieden *et al*, 1999; Ohashi *et al*, 1999; Eichler *et al*, 2003; Griffith, 2004]. Of note, the *in vitro* application of charybdotoxin and apamin blocks EDHF-mediated responses when selectively applied to the endothelium but not when applied to the adventitia [Doughty et al, 1999].

These peptides are toxic *in vivo* and therefore tetraethylammonium ion (TEA) has been employed as an inhibitor of K_{Ca} in clinical studies [Honing *et al*, 2000; Inokuchi *et al*, 2003; Bellien *et al*, 2005; Bellien *et al*, 2006; Pickkers *et al*, 2006;

Mortensen *et al*, 2007]. At concentrations of <1 mmol/L, TEA is a non-selective antagonist of vascular K_{Ca} [Nelson and Quayle, 1995; Ledoux *et al*, 2006] and, in bioassay experiments, it inhibits acetylcholine-mediated vasorelaxation of rabbit aortic endothelial rings when applied to the endothelium. Importantly, these effects are endothelium-specific and application to vascular smooth muscle that has been denuded of endothelium has no effect upon vascular tone [Demirel *et al,* 1994]. Furthermore, spectrofluorimetry studies reveal that TEA inhibits acetylcholine-induced elevation of intracellular Ca^{2+} concentration and patch-clamp recordings indicate that it inhibits the outward endothelial K^+ current [Demirel *et al*, 1994].

Tetraethylammonium ion attenuates forearm arterial vasodilatation induced by bradykinin [Honing *et al*, 2000] and substance P but does not affect vasodilatation in response to acetylcholine [Inokuchi *et al*, 2003]. This is in line with data from other groups demonstrating that, relative to bradykinin and substance P, acetylcholine does not evoke a strong NO-independent component for its endothelium-dependent vasodilator response [Shiramoto *et al*, 1997; Schrage *et al*, 2005]. Inokuchi *et al* report that TEA causes an increase in resting forearm arterial vascular tone [Inokuchi *et al*, 2003] although Honing *et al* report that it has no effect [Honing *et al*, 2000]. The reason for this discrepancy is unclear and warrants further investigation.

Putative Diffusible Mediators of EDHF

The role of K_{Ca} in the generation of endothelial hyperpolarisation is now well established but the identification of a single 'universal' mediator via which

endothelial hyperpolarisation may be transmitted to the underlying smooth muscle has proved to be substantially more challenging.

Nitric oxide and PGI_2 may both cause a degree of smooth muscle hyperpolarisation but there is extensive evidence to refute the hypothesis that EDHF simply represents 'residual' NO or PGI_2 remaining after the incomplete inhibition of NO synthase or cyclooxygenase [Illiano *et al*, 1992; Nagao *et al*, 1992; Griffith, 2004; Parkington *et al*, 2004]. Indeed, rather than augmenting EDHF, NO evokes a tonic inhibitory influence upon EDHF activity and EDHF-type activity remains after dual NO synthase/cyclooxygenase type 1 gene knockout [Bauersachs *et al*, 1996; Griffith, 2004; Vanhoutte, 2004; Scotland *et al*, 2005].

Many diffusible factors have been proposed to represent EDHF and include K^+ [Edwards *et al*, 1998; Dawes *et al*, 2002], hydrogen peroxide [Shimokawa *et al*, 1996; Matoba *et al*, 2000; Matoba *et al*, 2002; Matoba and Shimokawa, 2003; Miura *et al*, 2003; Griffith *et al*, 2004; Shimokawa and Matoba, 2004; Shimokawa and Morikawa, 2005], cytochrome P450 metabolites of arachidonic acid [Campbell and Harder, 1999; Fisslthaler *et al*, 1999; Quilley and McGiff, 2000; Halcox *et al*, 2001] and C-type natriuretic peptide [Chauhan *et al*, 2003; Chauhan *et al*, 2004]. However, none has consistently been shown to act as EDHF in all species and vascular beds and significant heterogeneity is observed between different experimental models, in the responses evoked by different pharmacologic agonists and in the presence or absence of interacting co-morbid conditions. Indeed, subsequent to the activation of vascular K_{Ca}, EDHF may represent the final common pathway of a number of

different mediators. As such, the term *endothelium-derived hyperpolarising factor* may be misleading and EDHF may represent a mechanism rather than a specific factor *per se*.

1.3 VASCULAR GAP JUNCTIONS

Gap junctions are found at points of cell-cell contact where they form an aqueous pore through which small hydrophilic molecules (<1 kDa) and ionic charge may pass. They are found in all tissues with the exception of skeletal muscle and non-nucleated cells such as red blood cells and platelets. They frequently aggregate to form 'plaques' at points of cellular contact where their characteristic pentalaminar structure may be seen under electron microscopy [Sandow and Hill, 2000]. Each gap junction comprises two hemichannels, or *connexons* that are composed of six *connexin (Cx)* subunits. The connexins traverse the cellular membrane four times and expose two extracellular peptide loops (Figure 1.2). Otherwise, they are a heterogeneous family classified by molecular weight in kDa and, although each connexon may be composed of a mix of connexin subtypes, Cx37, Cx40 and Cx43 (and occasionally Cx45) are particularly associated with mammalian endothelium and vascular smooth muscle [Christ *et al*, 1996; Figueroa *et al*, 2004; Sohl and Willecke, 2004]. The molecular structure of individual connexin subtypes is well conserved between species but large variation in their expression in both the endothelium and the smooth muscle exists between species, vascular bed and vessel size [Griffith, 2004].

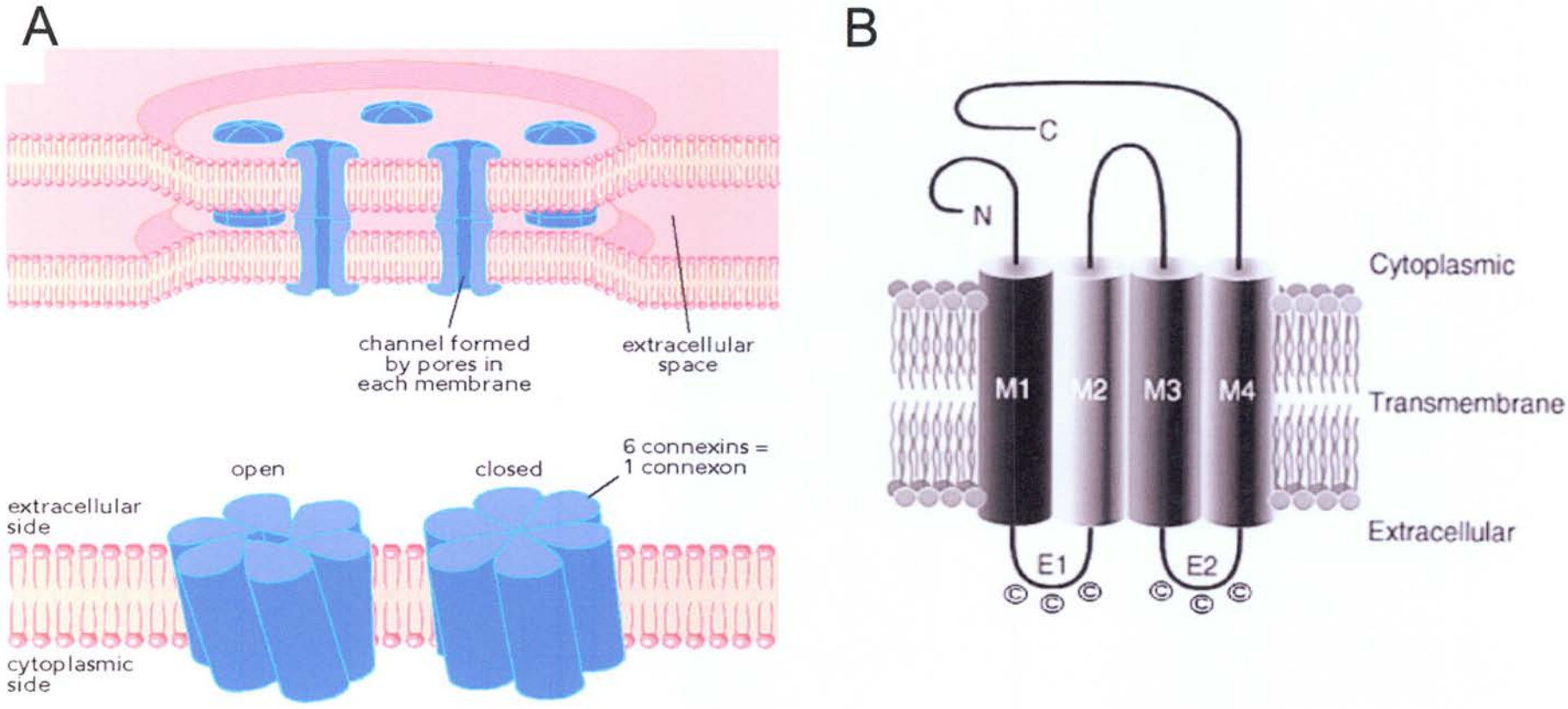


Figure 1.2 *(A) Schematic drawing of gap junction channels. Each apposed cell contributes a hemichannel to the complete gap junction channel. Each channel is formed by six protein subunits called connexins. Six connexin subunits of the hemichannel may simultaneously change configuration to open and close the hemichannel. Closure is achieved by connexin subunits sliding against each other and tilting at one end. (B) Topological model of a connexin. The cylinders represent transmembrane domains (M1 - M4). The loops between the first and the second, as well as the third and fourth transmembrane domains, are extracellular (E1 and E2, respectively) each with three conserved cysteine residues [Sohl and Willecke, 2004].*

1.3.1 REGULATION OF CONNEXIN EXPRESSION AND FUNCTION

Factors influencing connexin expression are poorly understood but physical influences may exert marked alterations of connexin expression, particularly Cx43. Cx43 expression is elevated by vascular shear stress [Gabriels and Paul, 1998; Depaola *et al*, 1999; Inai *et al*, 2004; Figueroa *et al*, 2006] and vascular injury [Yeh *et al*, 1997]. However, the effects of hypertension upon Cx43 expression are more variable and both an increase [Haefliger *et al*, 2006] and a decrease [Yeh *et al*, 2006] have been reported in hypertensive animal models. Connexin 43 expression also appears to be under the regulatory control of oestrogen – ovariectomy decreases Cx43 expression whilst oestrogen supplementation causes an increase [Liu *et al*, 2002; Chataigneau and Schini-Kerth, 2005]. The reported influence of hyperlipidaemia, statin treatment and inflammation upon the expression of various connexins has been variable [van Rijen *et al*, 1998; Sato *et al*, 2002; Yeh *et al*, 2003; Rignault *et al*, 2005]. Furthermore, expression of vascular connexin subtypes appears to be an interdependent phenomenon with connexin subtypes regulating the expression of others [Isakson *et al*, 2006].

Phosphorylation sites on the connexin COOH- terminus have been demonstrated and it is understood that both protein kinase A and C can affect connexin phosphorylation. Cyclic AMP increases gap junction permeability and, as such, metabolites of arachidonic acid may enhance communication via gap junctions [Griffith *et al*, 2002]. Additionally, phosphorylation may influence the assembly of connexons and modulate the permeability of the gap junction and the time that the connexin spends in residence at the gap junction before internalisation (usually in the

region of 2–5 hours). However, very little is known of how these processes influence *in vivo* function [Figueroa *et al*, 2004].

1.3.2 GAP JUNCTIONS AND EDHF

The cell membrane is impermeable to hydrophilic dyes such as Lucifer yellow or propidium bromide but they are small enough (<1 kDa) to pass through gap junctions and myoendothelial dye transfer via this route has been observed [Beny, 1999; Griffith, 2004]. Myoendothelial gap junctions are, therefore, ideally suited to the radial transfer of either electrotonic charge or one or more chemical mediators of hyperpolarisation from the endothelium to the underlying smooth muscle. Gap junctions would, therefore, permit specific and sensitive signalling and avoid the inevitable dilution associated with diffusible signalling molecules, such as NO.

Electrical myoendothelial coupling was initially suggested by bioassay experiments performed with 'sandwich' preparations in which the donor endothelium and detector vascular smooth muscle are uncoupled but closely apposed. In the presence of NO synthase inhibition, these vessels fail to demonstrate vasorelaxation in response to endothelium-dependent vasodilator agonists whilst intact 'electrically coupled' specimens relax normally [Chaytor *et al*, 1998; Griffith, 2004]. This raises the possibility that EDHF involves the passive electrotonic spread of hyperpolarisation via a contact mechanism. Indeed, myoendothelial gap junctions are perfectly located for this purpose and electrical coupling between the endothelium and smooth muscle has been demonstrated [Yamazaki and Kitamura, 2003]. Furthermore, gap junction plaques are most abundant in small resistance arteries and

their distribution is proportional to the magnitude of the EDHF-mediated response [Shimokawa *et al*, 1996; Sandow and Hill, 2000; Berman *et al*, 2002; Sandow *et al*, 2002].

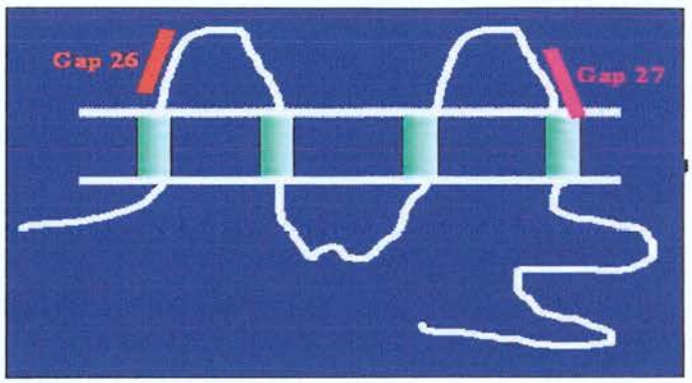
Observations from murine knockout models provide direct evidence for a role of gap junctions and specific connexins in the control of vascular tone. Mice lacking Cx40 are hypertensive [de Wit *et al*, 2003] and spontaneously hypertensive mice have a reduced expression of vascular Cx40 [Rummery *et al*, 2002; Rummery and Hill, 2004]. On the contrary, mice lacking endothelial Cx43 are hypotensive. However, these mice have a complex phenotype associated with a large elevation in plasma NO concentration [Liao *et al*, 2001]. It should also be noted that, in addition to being suited for the mediation of radial communication, gap junctions between adjacent homotypic smooth muscle or endothelial cells, provide a mechanism for the longitudinal conduction of vasomotion. Indeed, Cx40 deficient mice display irregular, uncoordinated vasomotion [de Wit *et al*, 2003].

The role of gap junctions in EDHF-mediated vascular responses *in vivo* in man remains unknown. Furthermore, the contribution of individual connexin subtypes to the mediation of EDHF responses remains unexplored in humans.

1.3.3 GAP JUNCTION BLOCKERS

Diverse agents have been employed as putative gap junction blockers. Of these, the anaesthetic halothane [Beny and Pacicca, 1994] and the liquorice derivative 18α-glycyrrhetinic acid have been most frequently used but they have other non-

specific EDHF-independent effects that limit their use [Chaytor *et al*, 2000; Matchkov *et al*, 2004]. In contrast, the connexin mimetic peptides (CMPs) are highly selective and specific gap junction inhibitors and permit assessment of individual connexin subtypes in EDHF-mediated vasodilatation [Dora *et al*, 1999; Evans and Boitano, 2001; Dhein, 2004]. These short synthetic peptides correspond to amino acid sequences in the first (Gap26) and second (Gap27) extracellular loops of Cxs37, 40 and 43 (Figure 1.3) and cause reversible disruption of connexin function and gap junction communication. Their exact mechanism of action remains unclear but they are thought to interfere with connexin gating properties [Evans and Boitano, 2001]. They do not suppress endothelial hyperpolarisation directly and do not exert non-gap junctional effects [Matchkov *et al*, 2006]. Unlike 18α-glycyrrhetinic acid, they do not affect the structural integrity, number or distribution of connexins [Berman *et al*, 2002].



	Gap26 Domain	Gap27 Domain
Cx37	VCYDQAFPISHIR	SRPTEKTIFII
Cx40	VCYDQAFPISHIR	SRPTEKNVFIV
Cx43	VCYDKSFPISHVR	SRPTEKTIFII

Figure 1.3 ***Top:*** *schematic representation of the connexin and the extracellular Gap26 and Gap27 regions to which the connexin mimetic peptides correspond.* ***Bottom:*** *amino acid sequences of Gap27 and Gap26 of Cx37, 40 and 43 (note homology). Adapted from Griffith, 2004[Griffith, 2004a] .*

In confluent COS fibroblasts expressing Cx43, intracellular transfer of Lucifer yellow is blocked by ^{37,43}Gap27 (SRPTEKTIFII) but not by ^{40}Gap27 (SRPTEKNVFIV) [Chaytor *et al*, 1999b]. Targeting CMPs against a single connexin subtype attenuates EDHF-mediated vasodilatation and the magnitude of this effect correlates with the relative expression of the connexin [Chaytor *et al*, 2005]. However, combined inhibition of the three predominant connexins generally produces more marked effects [Chaytor *et al*, 2003]. This not only reflects the heterogeneous expression of the connexins but also a propensity for the remaining connexins to compensate and maintain gap junction function [Griffith, 2004b].

Connexin mimetic peptides attenuate subintimal vascular smooth muscle hyperpolarisation and relaxation induced by acetylcholine, substance P and bradykinin in various *ex vivo* arterial and venous preparations from rabbit, rat, pig and guinea pig [Chaytor *et al*, 1997; Chaytor *et al*, 1998; Chaytor *et al*, 1999; Kwak and Jongsma, 1999; Chaytor *et al*, 2001; Berman *et al*, 2002; Sandow *et al*, 2002; Chaytor *et al*, 2003; Ujiie *et al*, 2003; Griffith *et al*, 2005]. Despite relatively large numbers of *ex vivo* studies using CMPs, concerns about the potential for systemic side-effects (for example, interference with gap junction-mediated cardiac conduction) have limited their use to just a handful of *in vivo* animal studies [De Vriese *et al*, 2002; Takenaka *et al*, 2008a; Takenaka *et al*, 2008b] and they have never been administered to man. In 2002, de Vriese *et al* examined the effects of the CMPs on NO and PGI_2-independent renal artery vasodilatation and blood flow in rats. With concurrent NO synthase and cyclooxygenase inhibition, bolus of ^{37,43}Gap27 into the renal artery partially inhibited the increase in blood flow evoked by acetylcholine whereas ^{40}Gap27 abolished the response entirely. Both peptides significantly decreased baseline renal blood flow and none of these effects were seen in response to a control 'scrambled' peptide [De Vriese *et al*, 2002].

1.3.4 Potentiators of communication via gap junctions

Gap junctions are distributed almost ubiquitously in the body and are particularly important in the coordinated conduction of the cardiac action potential. Therefore, the search for drugs that act to potentiate communication via gap junctions has been the topic of a substantial body of pharmaceutical research. Peptides with this property were first described in the 1980s [Aonuma *et al*, 1980] and were shown to

increase gap junction intracellular communication in the absence of changes in membrane conduction or basal current [Muller *et al*, 1997]. However, the clinical development of the original peptides was limited by their instability and very short half-life. Rotigaptide (ZP123) is a rotation-inversion of the original peptide that incorporates the unnatural D-configuration of the amino acids (Ac-D-Tyr-D-Pro-D-Hyp-Gly-D-Ala-Gly-NH_2) to provide much improved proteolytic stability [Kjolbye *et al*, 2003].

This novel hexpapetide antiarrhythmic agent has been safely administered to healthy humans [Udata *et al*, 2006] and is now in Phase II clinical trials [Kjolbye *et al*, 2003]. It prevents spontaneous ventricular [Xing *et al*, 2003; Hennan *et al*, 2006] and atrial [Shiroshita-Takeshita *et al*, 2007] arrhythmias in canine models of myocardial infarction and is effective in suppressing the development of atrial fibrillation in a canine model of mitral regurgitation [Guerra *et al*, 2006]. It promotes electrical coupling between ventricular myocytes by increasing gap junction conductance [Eloff *et al*, 2003; Clarke *et al*, 2006] via alterations in the phosphorylation status of Cx43 [Axelsen *et al*, 2006; Kjølbye *et al*, 2008] and it increases the number of gap junctions in the ischaemic myocardium [Hennan *et al*, 2006]. It exhibits no binding to a large array of receptors including numerous ion channels [Haugan *et al*, 2005] but potentiates gap junction-mediated dye transfer via Cx43 expressing HeLa cells but not via Cx26 or Cx32 [Clarke *et al*, 2006]. Rotigaptide's effects on electrical conduction and dye transfer via Cx37 and Cx40 have yet to be assessed.

Rotigaptide's effects upon resting vascular tone and endothelium-dependent agonist induced vasodilatation have not been examined. It remains unclear whether augmentation of communication via vascular gap junctions potentiates EDHF-mediated vascular actions.

1.4 ENDOGENOUS FIBRINOLYSIS

In addition to its central role in the control of vasomotion, the endothelium fulfils important fibrinolytic functions. The release of endogenous fibrinolytic factors protects the circulation from the propagation of intravascular fibrin deposition and thrombosis that would otherwise result in vascular occlusion and tissue ischaemia. In man, the principal endogenous fibrinolytic factor is tissue-type plasminogen activator (t-PA). This serine protease is stored in endothelial granules and, during the initiation of thrombus formation, its acute calcium-dependent release is stimulated by members of the clotting cascade including thrombin and factor Xa [van den Eijnden-Schrauwen et al, 1997]. In the circulation, t-PA catalyses the conversion of plasminogen to plasmin and the ensuing enzymatic process results in the cleavage of fibrin with consequent generation of fibrin degradation products [Cruden and Newby, 2005].

The fibrinolytic efficacy of t-PA is inversely proportional to the circulating concentration of its major serpin inhibitor, plasminogen activator inhibitor type 1 (PAI-1), with which t-PA forms an inactive complex [Oliver *et al*, 2005] (Figure 1.4).

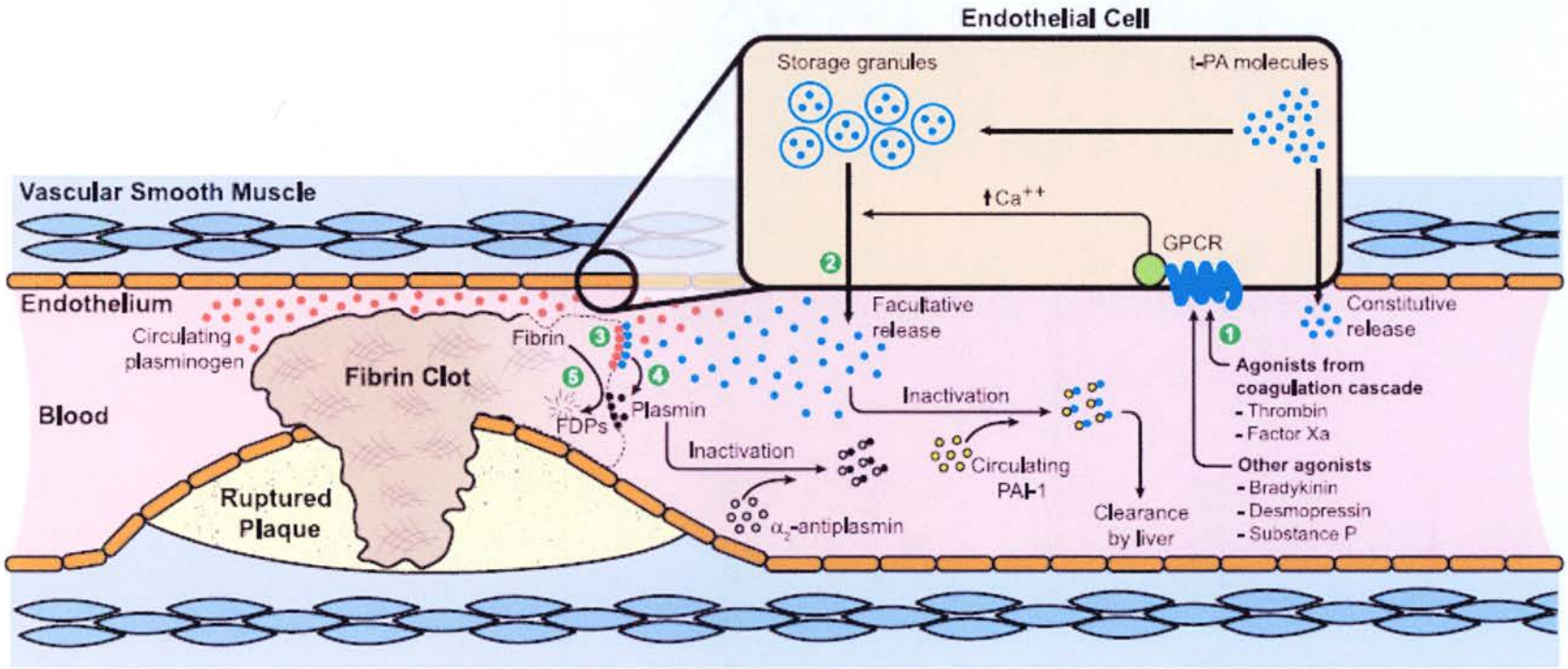


Figure 1.4 *The endothelial fibrinolytic response to luminal thrombus. Agonists generated from the coagulation cascade act on endothelial cell surface G-protein coupled receptors (GPCRs) (1) to stimulate release of tissue plasminogen activator (t-PA) from stoage granules, a step that requires an increase in intracellular calcium (Ca^{2+}) concentration (2). Free t-PA acts on thrombus-bound plasminogen (3) to produce plasmin (4) that, in turn, degrades cross-linked fibrin into fibrin degradation products (FDPs) (5), thus dissolving the thrombus. The fibrinolytic process is inhibited by inactivation of t-PA by plasminogen activator inhibitor type 1 (PAI-1) and plasmin by α2-antiplasmin [Oliver et al 2005].*

1.4.1 ENDOTHELIAL MECHANISMS OF T-PA RELEASE

In addition to stimulation by members of the coagulation cascade, a variety of other agonists evoke the endothelial release of t-PA *in vivo* in man. Of these, bradykinin is the best characterised *in vivo* – acting via the B_2 kinin receptor, it causes marked t-PA release that is not attenuated by inhibition of NO and PGI_2 generation [Brown *et al*, 2000]. Similarly, both adenosine and uridine triphosphate evoke the release of t-PA in the human forearm via a NO- and PGI_2-independent mechanism [Hrafnkelsdottir et al, 2001]. As such, it has been suggested that EDHF may be responsible for their induction of t-PA release. Smith *et al* observed that the inhibition of NO synthesis augments bradykinin-induced t-PA release [Smith *et al*, 2003] and it is conceivable that, in the absence of NO, EDHF may become up-regulated with consequential augmentation of endogenous fibrinolytic activity. Consistent with this hypothesis, elevated basal concentrations of t-PA are found in a genetically engineered murine model lacking eNOS [Iafrati *et al*, 2005].

Although it has been demonstrated that relatively prolonged intra-arterial infusion of acetylcholine causes the release of t-PA in the human forearm [Giannarelli *et al*, 2007], the majority of studies to examine the effects of intra-arterial acetylcholine have not demonstrated any effect upon t-PA release [Brown *et al*, 1999; Minai *et al*, 2001; Chia *et al*, 2003]. Similar to acetylcholine, methacholine is an agonist at muscarinic receptors but, in contrast, it consistently evokes t-PA release [Stein *et al*, 1998; Pretorius *et al*, 2002]. Interestingly, acetylcholine mediates forearm arterial vasodilatation almost entirely via the NO pathway. This contrast with bradykinin,

methacholine, ATP and UTP which act via EDHF to a greater extent [Chowienczyk *et al*, 1993; Hrafnkelsdottir *et al*, 2001; Pretorius *et al*, 2002].

It is of note that NO donors such as sodium nitroprusside do not evoke t-PA release despite causing potent arterial vasodilatation. This evidence has been used to further argue against a direct role for NO in the induction of t-PA release. However, the argument is complicated by the observation that substance P-induced t-PA release is attenuated by NO synthase inhibition [Newby *et al*, 1998]. Nitric oxide may, therefore, exert a permissive role upon substance P-induced t-PA release but this effect is not a universal feature and may be agonist-specific.

The observation that NO donors evoke vasodilatation without associated t-PA release also provides evidence that endothelial t-PA release is not simply a flow-related phenomenon. This finding is further reinforced in the observation that intra-arterial tumour necrosis factor-alpha (TNF-α) evokes t-PA release in the forearm circulation but it attenuates endothelium-dependent vasodilatation [Chia *et al*, 2003] and norepinephrine induces t-PA release in association with vasoconstriction [Jern *et al*, 1994; Oliver *et al*, 2005].

Thus, endothelial mechanisms underlying t-PA release are complex and incompletely understood but may be either mediated or influenced by EDHF, or substances related to this mechanism. Indeed, Muldowney and colleagues examined mechanisms of thrombin-mediated t-PA release from cultured endothelial cells. They demonstrated that, although thrombin-mediated release of t-PA *in vitro* is not blocked by inhibitors

of NO or PGI_2 synthesis, it is abolished by inhibition of the generation of 5,6-epoxyeicosatrienoic acid (5,6-EET) [Muldowney *et al*, 2007]. Although there is substantial evidence that EETs contribute to the EDHF mechanism [Feletou and Vanhoutte, 2006a], it should be noted that inhibitors of K_{Ca} did not influence t-PA release in this model and any extrapolation of findings from this *in vitro* study to the *in vivo* situation should be cautious.

A direct assessment of the role of EDHF in human t-PA release *in vivo* has not been made. Specifically, the role of K_{Ca} and gap junctions in this process remains unknown.

1.5 THROMBIN

Thrombin (factor IIa) is the principal effector protease of the coagulation cascade (Figure 1.5) and is an integral mediator both in vascular physiology and in pathophysiology. In addition to its pivotal procoagulant role it displays other important vascular haemostatic, vasomotor and inflammatory properties [Coughlin, 2000; Coughlin, 2005].

Thrombin generation is triggered when vascular integrity is disrupted and plasma coagulation factors are allowed contact with extra-vascular tissue factor. It causes the conversion of circulating fibrinogen to fibrin monomer which polymerises to form fibrin, the fibrous matrix of blood clots (Figure 1.5). Additionally, thrombin acts as a potent cellular stimulant – it is the most powerful platelet activating factor known

[Gailani and Renné, 2007], it evokes smooth muscle proliferation [Koenig, 2003] and it causes vascular hyperpermeability and oedema [Lum and Malik, 1994]. By inducing the expression of chemokines and endothelial adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), P-selectin and von Willebrand factor (vWF), it causes increased recruitment of platelets and leucocytes to the endothelial surface [Coughlin, 2005]. Via these actions, it acts not only as an essential inflammatory and haemostatic factor localising appropriate repair to sites of vascular injury, but can also be deleterious and is central to the pathophysiology of atherothrombosis [Croce and Libby, 2007].

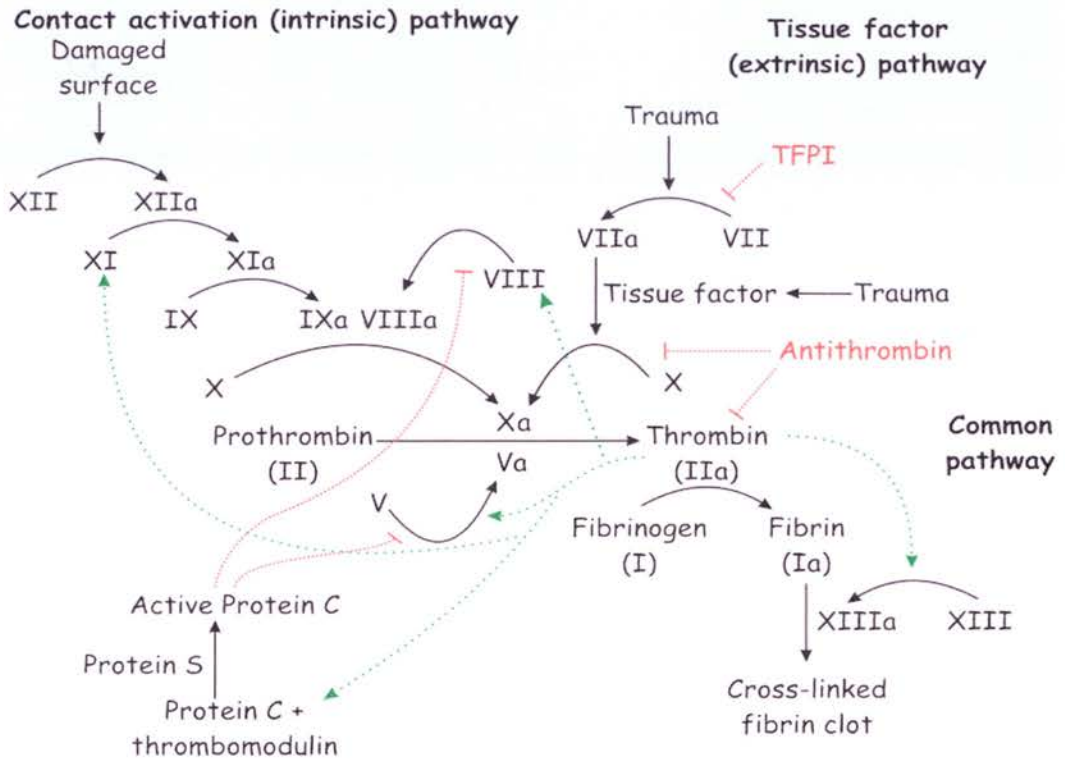


Figure 1.5 *Coagulation cascade. Inhibitors of coagulation denoted by red dotted lines; positive feedback denoted by green dotted lines (TFPI=tissue factor pathway inhibitor).*

1.5.1 VASCULAR PROTEASE-ACTIVATED RECEPTORS

An extensive search for a receptor responsible for thrombin's extra-coagulant cellular actions culminated in the discovery and cloning of protease-activated receptor type 1 (PAR-1) [Vu *et al,* 1991]. Subsequently, three further protease-activated receptors have been characterised: PAR-2, -3 and -4. PAR-1, -3 and -4 are all activated by thrombin but PAR-2 is principally activated by trypsin [Ossovskaya and Bunnett, 2004]. Protease-activated receptor type 1 is the principal factor IIa receptor in man and is activated by the presence of thrombin at sub-nanomolar concentrations by virtue of a hirudin-like sequence that resides in the N-terminal extracellular domain of PAR-1, but not PAR-4 which is activated by higher concentrations of thrombin [Ossovskaya and Bunnett, 2004; Leger *et al*, 2006]. Protease-activated receptor type 3 is a high affinity thrombin receptor in animals other than primates [Leger *et al*, 2006]. All belong to the seven transmembrane α-helical receptor superfamily and are characterised by a unique mechanism of action. Firstly, proteolytic cleavage of the amino-terminal extracellular domain exposes a 'cryptic' ligand that remains attached to the N-terminal domain of the protease-activated receptor. This tethered ligand subsequently undergoes intramolecular rearrangement to allow ligand and receptor moieties to interact (Figure 1.6) to evoke their biological effects via G-protein coupling. Once activated, PAR is rapidly uncoupled from G-protein mediated signalling and internalised in lysosomes for degradation [Vu *et al*, 1991; Coughlin, 2000; Barnes *et al*, 2004].

Protease-activated receptor type 1 is expressed on cardiomyocytes, smooth muscle cells, the vascular endothelium, fibroblasts and on platelets as well as in many locations outside the vasculature [Ossovskaya and Bunnett, 2004]. It is of note, however, that murine and rat platelets do not express PAR-1 *[Kinlough-Rathbone et al*, 1993; Derian *et al*, 1995] and consequently studies of PAR-1 in these animals are not always of direct relevance to man.

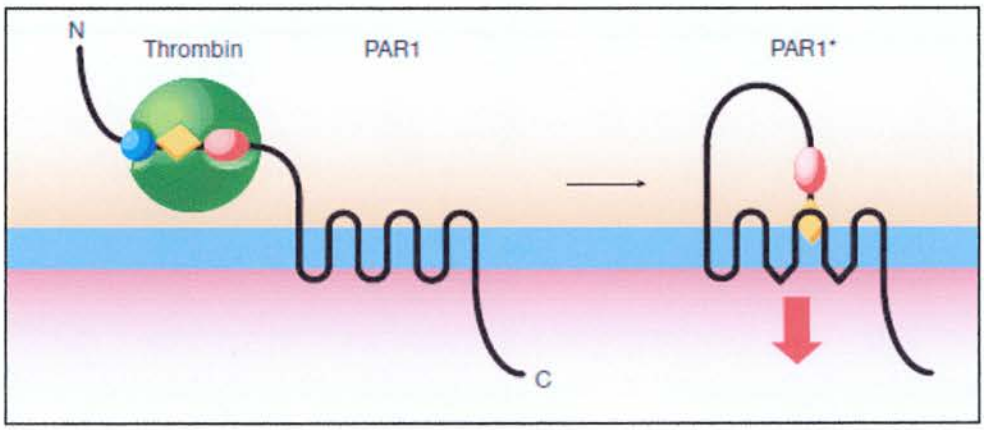


Figure 1.6 *Mechanism of protease-activated receptor type 1 (PAR-1) activation. Thrombin (large green sphere) recognises the N-terminal domain of the G-protein coupled thrombin receptor PAR-1. This interaction uses sites at N-terminal (small blue sphere) and C-terminal (small pink oval) to the thrombin cleavage site. Thrombin cleaves the peptide bond between receptor residues Arg 41 and Ser 42. This serves to unmask a new N terminus, beginning with the sequence SFLLRN (diamond) that functions as a tethered ligand, docking with the body of the receptor to effect transmembrane signalling. From Coughlin, 2000 [Coughlin, 2000].*

PAR-1 Activating Peptide

Proteolytic cleavage of the N-terminal domain of PAR-1 by thrombin reveals the amino acid sequence, SFLLRN, which is the active, tethered ligand that causes autoactivation of the receptor [Vu *et al*, 1991]. Similarly, synthetic exogenous SFLLRN acts directly upon PAR-1 without the requirement for proteolytic cleavage of the receptor but otherwise mimics the cellular actions of thrombin [Scarborough *et al*, 1992]. Indeed, SFLLRN has been used extensively as a PAR-1 activating peptide and is a valuable tool for the examination of PAR-1 and thrombin's cellular effects in the vasculature. By use of the PAR-1 activating peptide, the cellular effects of thrombin can be examined without the confounding and potentially harmful effects of potent stimulation of the coagulation cascade [Coughlin, 2005].

PAR-1 Antagonists

Inhibition of PAR-1 mediated actions has captured the interest of the pharmaceutical industry. The development of a drug with the potential to antagonise the cellular effects of thrombin without affecting its actions in the coagulation cascade could represent an important antithrombotic treatment strategy that may be associated with less bleeding complications than conventional antithrombin inhibitors [Chackalamannil, 2006]. Indeed, a PAR-1 antagonist (SCH 530348) has displayed these properties in a phase II clinical trial and is currently being evaluated in phase III studies [Husted, 2007].

Due to the diverse cellular actions of thrombin including cellular proliferation and inflammation and the widespread expression of PAR-1, these antagonists have also

been proposed for the treatment of restenosis, atherosclerosis, inflammation, [Chackalamannil, 2006] stroke [Olson *et al*, 2004] and non-vascular conditions including cancer metastasis [Kaufmann *et al*, 2007] and neurodegeneration [Hamill *et al*, 2007]. Therefore, the examination of PAR-1 mediated effects is pertinent not only to our understanding thrombin's cellular effects in physiology and pathophysiology, but is also important to inform the clinical development of PAR-1 antagonists.

Vascular Effects of PAR-1 Activation

In vitro activation of PAR-1 causes endothelium-dependent vasorelaxation in various animal arterial preparations [Kawabata *et al*, 2004a; Kawabata *et al*, 2004b] and in human coronary [Hamilton *et al*, 1998] and pulmonary [Hamilton *et al*, 2001b] arteries. Furthermore, systemic intravenous administration of SFLLRN to rodents culminates in a sustained drop in blood pressure that is attenuated by L-NAME [Sicker et al, 2001]. In addition to a requirement for NO, EDHF appears to make a large contribution to PAR-1 induced arterial vasodilatation. Kawabata and colleagues demonstrated that rat gastric arterial vasodilatation induced by PAR-1 activating peptide is markedly reduced by the K_{Ca} antagonists, charybdotoxin and apamin, whilst the further addition of antagonists of NO and PGI_2 production abolished the response. They characterised the EDHF component of the vasodilator response further and found no role for cytochrome P450 metabolites of arachidonic acid or for hydrogen peroxide. However, the putative gap junction blockers carbenoxolone and 18α-glycyhrretinic acid significantly attenuated the EDHF-mediated response

[Kawabata *et al*, 2004b]. In contrast, activation of smooth muscle PAR-1 causes arterial vasoconstriction [Ku and Dai, 1997].

Our group has recently, and for the first time, demonstrated that PAR-1 activation *in vivo* in healthy human subjects causes arterial vasodilatation, venous constriction, t-PA release and platelet activation [Guðmundsdóttir *et al*, 2006]. It has been proposed that, acting via PAR-1, thrombin evokes these vascular responses as a protective feedback mechanism in an attempt to maintain vessel patency. In the presence of developing thrombus, arterial PAR-1 activation would cause arterial vasodilatation and increase the release of t-PA to ensure rapid clearance and clot dissolution. As veins do not have resting tone venous dilatation would not be beneficial but venoconstriction would potentially limit thrombus propagation and embolisation [Guðmundsdóttir *et al*, 2006]. The role of the endothelium and endothelium-derived factors including NO, PGI_2 and EDHF in these responses remains unknown.

1.6 ENDOTHELIAL DYSFUNCTION

The formation of atherosclerosis is a complex inflammatory process that involves a series of interactions with many cell types including the endothelium [Hansson, 2005]. As well as a wide variety of disease states now known to predispose to atherosclerosis, classical 'risk factors' including smoking [Newby *et al*, 1999; Newby *et al*, 2001; Pretorius *et al*, 2002; Takashima *et al*, 2007], hypercholesterolaemia [Celermajer *et al*, 1992; Stroes *et al*, 1995], diabetes mellitus

[Calver *et al*, 1992], hypertension [Lauer *et al*, 2005] and a family history of premature vascular disease [Celermajer et al, 1992] are all associated with endothelial dysfunction [Félétou and Vanhoutte, 2006b]. Furthermore, the disruption of endothelial haemostatic mechanisms increases the risk of reduced blood flow, local thrombus formation and consequent vessel occlusion and tissue infarction.

1.6.1 ENDOTHELIAL DYSFUNCTION AND VASOMOTION

The assessment of endothelial function has been performed principally via the examination of endothelium-dependent vasodilatation. The response to endothelial stimulants is often examined in the peripheral resistance vasculature [Wilkinson and Webb, 2001] and the response to both pharmacologic stimulation and to reactive hyperaemia is measured in conduit vessels [Felmeden and Lip, 2005]. Similar to the diminished peripheral arterial endothelium-dependent vasodilation seen in patients with atherosclerosis and its risk factors, endothelium-dependent vasodilator responses are also impaired in the coronary arterial circulation of these subjects [Ludmer *et al*, 1986; Zeiher *et al*, 1995a; Zeiher *et al*, 1995b; Kugiyama *et al*, 1996]. Therefore, it is proposed that impaired endothelial responses are a systemic feature of atherosclerosis and its risk factors.

Furthermore, impaired endothelium-dependent vasomotor dilatation in the peripheral [Heitzer *et al*, 2001; Perticone *et al*, 2001] and coronary [Suwaidi *et al*, 2000; Halcox *et al*, 2002] circulations independently predicts the risk of future adverse cardiovascular events even in subjects without angiographic evidence of significant coronary disease [Suwaidi *et al*, 2000].

1.6.2 NITRIC OXIDE, EDHF AND ENDOTHELIAL DYSFUNCTION

Although endothelial dysfunction occurs in many different disease states, oxidative stress is the common pathophysiological denominator [Félétou and Vanhoutte, 2006b]. The synthesis and activity of NO is particularly susceptible to oxidative stress but EDHF appears to be relatively resistant [Kaw and Hecker, 1999]. Indeed, EDHF frequently acts as a back-up mechanism, undergoing up-regulation in the face of reduced NO bioavailability in a variety of disease states including heart failure [Malmsjo *et al*, 1999; Katz and Krum, 2001], coronary artery disease [Miura *et al*, 2003], type II diabetes mellitus [Triggle *et al*, 2004] and after ischaemia/reperfusion [Marrelli, 2002] and angioplasty [Thollon *et al*, 2002]. The effect of hypercholesterolaemia upon EDHF-mediated responses remains unclear with both an increase [Brandes *et al*, 1997] and decrease [Urakami-Harasawa *et al*, 1997] in its activity reported. Similarly, the role of EDHF in the cause and consequences of hypertension remains unclear with both increased [Taddei *et al*, 2006] and decreased [Fujii *et al*, 1993] activity described in different models. Conditions specifically associated with impairment of EDHF-mediated responses are in the minority but include type I diabetes mellitus [Fukao *et al*, 1997; Wigg *et al*, 2001; Angulo *et al*, 2003] and pre-eclampsia [Kenny *et al*, 2002]. Whilst cigarette smoking primarily affects NO bioavailability [Ambrose and Barua, 2004], its effect upon EDHF activity has not been assessed *in vivo*. However, a small *in vitro* study of rat mesenteric and human middle cerebral arteries exposed to lipid soluble smoke particles revealed a concomitant attenuation of both NO and EDHF-mediated vasodilatation [Zhang et al, 2006].

1.6.3 ENDOTHELIAL DYSFUNCTION AND ENDOGENOUS FIBRINOLYSIS

The assessment of endothelium-dependent vasomotion has been the mainstay of the *in vivo* assessment of endothelial function. However, endothelium-dependent vasomotion may not be representative of other important endothelial functions such as the regulation of endogenous fibrinolysis.

In the absence of appropriate endothelial release of t-PA, subclinical microthrombi that would normally undergo lysis may be given the opportunity to propagate and cause vessel occlusion and tissue infarction [Oliver *et al*, 2005]. Indeed, a reduction in basal t-PA activity or release is associated with an increased incidence of major cardiovascular events in patients with stable [Held *et al*, 1997] and unstable [Hoffmeister *et al*, 1995] angina.

The measurement of resting t-PA concentrations is of interest but the assessment of the endothelium's capacity for the dynamic release of t-PA is more pathophysiologically relevant and not reflected by basal concentrations [Hrafnkelsdottir *et al*, 2004a]. Indeed, efficient fibrinolysis requires the local and timely release of t-PA stimulated by the presence of a developing thrombus [Fox *et al*, 1985]. In patients with cardiovascular disease our group has recently demonstrated that impaired release of pharmacologically stimulated t-PA predicts the risk of future adverse cardiovascular events [Robinson *et al*, 2007].

Acute coronary t-PA release is inversely proportional to the atherosclerotic burden [Newby et al, 2001]. Furthermore, t-PA release stimulated by intra-arterial substance P or bradykinin is impaired in the coronary and peripheral circulation of smokers [Newby *et al*, 1999; Newby *et al*, 2001; Pretorius *et al*, 2002; Takashima *et al*, 2007]. Of note, the impairment of stimulated t-PA release found in smokers [Pretorius *et al*, 2002] and patients with hypertension [Hrafnkelsdottir *et al*, 1998; Hrafnkelsdóttir *et al*, 2004b] has been reported in the absence of a change in endothelium-dependent vasomotor responses. Therefore, in some circumstances the assessment of acute stimulated t-PA release may represent a more sensitive marker of endothelial function. Conversely, patients with hypercholesterolaemia display impaired endothelium-dependent vasomotor responses without any alteration in the capacity for the acute stimulated release of t-PA [Newby *et al*, 2002]. Not only does this underline the complexity of vascular endothelial function, but it also reinforces the requirement for broader assessment of endothelial functions without undue reliance upon a single surrogate marker of endothelial health.

1.6.4 PAR-1 ACTIVATION AND THE ASSESSMENT OF ENDOTHELIAL FUNCTION

Healthy excised human coronary arteries dilate in response to PAR-1 activation but, in those with severe atherosclerosis, this response is either absent or marked vasoconstriction occurs [Ku and Dai, 1997]. In the context of endothelial dysfunction, the loss of protective arterial dilatation could have marked effects upon the delicate balance dictating whether a developing thrombus undergoes successful fibrinolysis or expands to cause clinically relevant vessel occlusion.

The dynamic *in vivo* assessment of regional t-PA release has employed the intra-arterial administration of a variety of pharmacologic stimulants, most frequently in the forearm and coronary circulations. Of these stimulants, our group and others have most frequently used substance P and bradykinin [Newby *et al*, 1997a; Newby *et al*, 2001; Pretorius *et al*, 2002; Takashima *et al*, 2007]. However, although substance P has been a useful pharmacologic tool, it is a neurotransmitter and evidence to suggest that it plays any role as a pathophysiological mediator in atherothrombosis is limited. 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].

Given its central role in thrombosis and inflammation, thrombin is the most powerful and pathophysiologically relevant mediator of atherothrombosis [Coughlin, 2005]. However, safety concerns, particularly with regard to its potent procoagulant effects, have precluded its direct intra-arterial administration in man. However, by intra-arterial administration of SFLLRN, the examination of PAR-1 responses allow the assessment of thrombin's cellular effects in the absence of activation of the coagulation cascade. Such a pathophysiologically relevant and potentially more sensitive assessment of endothelial function *in vivo* in subjects with atherosclerosis and its risk factors has previously not been made.

1.7 AIMS

The aims of this thesis were:

In human subcutaneous resistance arteries (Chapter 3):

- To clarify the relative contributions of NO, PGI_2 and EDHF to endothelium-dependent vasodilatation.
- To determine whether EDHF-mediated vasodilatation can be abolished using a combination of CMPs.
- To establish the presence of the three major vascular connexin subtypes.
- To determine whether any of the three major vascular connexins contributes to the EDHF mechanism to a relatively greater extent than the others.

In isolated rabbit myocardium (Chapter 4):

- To establish the biological activity of rotigaptide.

In healthy volunteers (Chapter 4):

- To establish, using intra-arterial rotigaptide, whether basal forearm vascular tone, agonist-induced vasodilatation and t-PA release can be augmented by increasing communication via gap junctions containing connexin 43.
- To establish whether rotigaptide specifically augments EDHF-mediated vasodilatation.

In healthy volunteers (Chapter 5):

- To establish the role of the endothelium, and endothelial factors including NO, PGI_2 and EDHF in the vascular effects of PAR-1 activation *in vivo.*
- To clarify that EDHF-mediated actions can be inhibited *in vivo* by antagonism of calcium-activated potassium channels using TEA.
- To establish whether EDHF is responsible for bradykinin and PAR-1 activation-induced t-PA release.

In otherwise healthy volunteers who smoke (Chapter 6):

- To establish the effect that smoking cigarettes has upon vascular endothelial responses to PAR-1 activation.

1.8 HYPOTHESES

The following hypotheses will be addressed:

Connexins, gap junctions and their involvement in the EDHF mechanism:

1. Gap junction blockade with connexin mimetic peptides inhibits EDHF-mediated vasorelaxation in pregnant human subcutaneous resistance arteries.
2. Connexins 37, 40 and 43, the three principal vascular connexins, are expressed in pregnant human subcutaneous resistance arteries.
3. Potentiation of communication via gap junctions with rotigaptide enhances EDHF-mediated actions to increase human forearm arterial basal blood flow, agonist-induced vasodilatation and endothelial t-PA release.

PAR-1 activation and endothelial responses:

1. Activation of PAR-1, the principal vascular thrombin receptor, causes vasodilatation via the endothelial release of NO, PGI_2 and EDHF in the human forearm arterial circulation.
2. PAR-1 activation evokes t-PA release via EDHF in the human forearm arterial circulation.
3. Endothelial dysfunction caused by smoking cigarettes results in an impairment of PAR-1 mediated vasodilatation and t-PA release in the human forearm arterial circulation.

CHAPTER 2

METHODOLOGY

2.1 INTRODUCTION

Many techniques have been developed and refined for the examination of vascular responses both *in vitro* and *in vivo.* An overview of the techniques employed in this thesis is presented below. Details specific to each study can be found in the methods section of subsequent chapters.

2.1.1 MYOGRAPHY

Vascular responses can be examined in isolated vessels using myography. This widely used technique is very useful for the assessment of novel compounds before assessment of their *in vivo* effects and for the assessment of compounds with the potential for systemic toxicity. The arrangement allows for precise changes to be made to the controlled environment of the organ bath.

Myography has been used extensively to assess the contribution and mechanism of EDHF in vasorelaxation [Griffith, 2004; Chaytor *et al*, 2005; Mackenzie *et al*, 2008]. The vast majority of these assessments have been made in vessels obtained from animals and in a wide range of vascular beds. However, the EDHF response is particularly variable between vascular bed, vessel size and species making comparisons of results difficult [Griffith, 2004]. Indeed, direct examination of the role of gap junctions and connexin subtypes in EDHF-mediated responses in the human vasculature had not previously been made. In this thesis, human subcutaneous resistance arteries were used to make this assessment for the first time. Other aspects of the pharmacology of these vessels have been examined in some depth previously

and indicate a large EDHF-mediated component to their vasorelaxant mechanism [Coats *et al*, 2001; McIntyre *et al*, 2001; Luksha *et al*, 2004].

Furthermore, segments from the same vessels examined using myography can be preserved for the immunohistochemical visualisation of relevant receptors or protein constituents, including the vascular connexins.

2.1.2 FOREARM VENOUS OCCLUSION PLETHYSMOGRAPHY

Whilst myograpy provides a precisely controlled environment *in vitro,* bilateral forearm venous occlusion plethysmography allows a direct assessment of peripheral vascular responses *in vivo.* Coupled with unilateral brachial artery infusion, it is a powerful and reproducible technique that allows the direct assessment of forearm vascular responses to agonists and antagonists at doses 10- to 1000-fold lower than required to evoke a systemic effect. This dose reduction allows a reduction in the risk of unwanted or potentially harmful side-effects and simultaneously minimises the possibility of confounding cardiac, renal and neural reflexes that can occur with systemic dosing [Benjamin *et al*, 1995; Wilkinson and Webb, 2001].

Venous occlusion plethysmography relies upon the intermittent prevention of venous drainage from the arm. This is achieved using upper arm cuffs inflated above venous, but not arterial, pressure. Thus, blood is allowed to enter the forearm normally but cannot escape. This causes a linear increase in forearm volume over time, which is proportional to arterial blood inflow. Under resting conditions, approximately 70% of total forearm blood flow (FBF) is through skeletal muscle and the measured

vascular response is predominantly a reflection of flow through the forearm resistance arterial system. It is, therefore, a particularly useful technique for the assessment of EDHF-mediated responses. As the hand contains a high proportion of arteriovenous shunts with a different pharmacology and physiology, it is excluded from the circulation by the application of inflation cuffs at suprasystolic pressure during FBF [Wilkinson and Webb, 2001].

The technique of bilateral FBF measurement is highly reproducible within individuals [Walker *et al*, 2001; Wilkinson and Webb, 2001] and can be combined with intermittent venous sampling for the measurement of plasma fibrinolytic factors [Newby *et al*, 1997a].

2.1.3 AELLIG DORSAL HAND VEIN TECHNIQUE

The assessment of venous compliance in a single dorsal hand vein using the Aellig displacement technique permits the direct effect of locally active, subsystemic doses of infused drugs to be examined without systemic influences [Aellig, 1981; Aellig, 1994a; Aellig, 1994b]. In addition to allowing the *in vivo* assessment of venomotor responses, it allows direct assessment of the role of the endothelium in these responses without recourse to pharmacologic blockade of endothelium-derived factors. Whilst it is not possible to safely remove the endothelium from the arterial circulation, the endothelium lining a short segment of the dorsal hand vein can be disrupted *in vivo* via the instillation of distilled water [Collier and Vallance, 1990; Sogo *et al*, 2000].

2.1.4 ELECTROPHYSIOLOGIC OPTICAL MAPPING

The putative gap junction potentiating peptide, rotigaptide, has been developed as an antiarrhythmic agent. Therefore, it has a body of complementary electrophysiologic data to support its efficacy in this respect [Haugan *et al*, 2005a; Haugan *et al*, 2005b; Guerra *et al*, 2006; Haugan *et al*, 2006; Hennan *et al*, 2006; Shiroshita-Takeshita *et al*, 2007]. Custom-synthesised, pharmaceutical grade rotigaptide was obtained for use in the clinical vascular studies but before application in these studies, its biological activity was verified using an electrophysiologic optical mapping system [Bayly *et al*, 1998; Walker *et al*, 2007].

2.2 GENERAL

2.2.1 ETHICAL CONSIDERATIONS

All human studies were undertaken in accordance with the Declaration of Helsinki and with the approval of the local Research Ethics Committee. The written informed consent of each subject was obtained before entry into the study.

2.2.2 SUBCUTANEOUS FAT BIOPSIES

Subcutaneous fat biopsies were obtained from healthy women undergoing elective caesarean section following uncomplicated pregnancy. Biopsies were taken from the incision margin and resistance arteries subsequently dissected out. Biopsies were not taken from women with a history of diabetes mellitus, hypertension or any other significant past medical history.

2.2.3 PERFUSED RABBIT LEFT VENTRICULAR WEDGE PREPARATION

Male New Zealand white rabbits were killed with a single intravenous injection of 100 mg/kg pentobarbital sodium and their hearts subsequently excised. These procedures conform to the standards set out in the Animals (Scientific Procedures) Act 1986.

2.2.4 SUBJECT PREPARATION

In the *in vivo* studies, none of the volunteers received vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study. All subjects and patients abstained from alcohol for 24 hours and from food, tobacco and caffeine-containing drinks for at least 4 hours prior to the study. Studies were performed in a quiet, temperature controlled room maintained at 22-25°C.

2.2.5 BLOOD PRESSURE MEASUREMENT

During forearm venous occlusion plethysmography and dorsal hand vein studies, blood pressure was monitored in the non-infused arm at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan).

2.3 MYOGRAPHY

2.3.1 TISSUE PREPARATION

Subcutaneous fat biopsies were taken from the caesarean section incision margin and immediately placed in cold physiological salt solution (PSS) (NaCl 119 mmol/L,

KCl 4.7 mmol/L, $CaCl_2$ 2.5 mmol/L, $MgSO_4$ 1.17 mmol/L, $NaHCO_3$ 25 mmol/L, KH_2PO_4 1.18 mmol/L, EDTA 0.026 mmol/L, glucose 5.5 mmol/L). Resistance arteries (approximate internal diameter 100–300 μm) were dissected free of surrounding fat and connective tissue, and cut into segments approximately 2 mm long. All arteries were kept in PSS at 4°C and used on the day of biopsy.

2.3.2 Changes in vessel tension

Arterial segments were mounted in the organ baths of a four-channel wire-myograph (multimyograph, model 610, Danish Myo Technology; Aarhus, Denmark). Two stainless steel wires (25 μm in diameter) were threaded through the lumen of each artery segment. One wire was attached to a force transducer and the other to a micropositioner in order to measure vessel tension. Each organ bath contained warmed (37°C) PSS and was continuously bubbled with 5% carbon dioxide and 95% oxygen. Following a 30-minute equilibration period, a passive circumference-tension curve was created for each segment in order to set optimum resting tension. This resting tension is calculated to simulate an *in vivo* transmural pressure of 100 mmHg. Arteries were then set at 90% of this tension to enable optimal contractile conditions with a low resting tension [Mulvany and Halpern, 1976]. This semi-automated procedure also allows the calculation of arterial diameter. All solutions were refreshed every 30 minutes.

Arterial segments were caused to constrict five times as part of a standard 'start procedure' [Aalkjaer *et al*, 1987]. The first, second and fifth contractions were evoked by high (124 mmol/L) potassium solution (KPSS; made by equimolar

substitution of KCl for NaCl in PSS) containing 10 μmol/L norepinephrine. The third and fourth were obtained with 10 μmol/L norepinephrine or KPSS alone. Arteries that failed to produce constriction equivalent to 100 mmHg when constricted with KPSS containing 10 μmol/L NE were rejected.

Prior to the addition of vasodilators, constriction was elicited using norepinephrine (3 μmol/L). Endothelial viability was assessed by the addition of a single dose of bradykinin (1 μmol/L) to each chamber. Arteries that did not achieve at least 60% vasorelaxation to bradykinin were excluded.

2.3.3 Data analysis

Calibration and data processing were performed using Myodac software (version 2.1, Danish Myo Technology). Vasorelaxation is expressed as a percentage of the return to basal tone produced by the highest concentration of bradykinin (3 μmol/L) after preconstriction with norepinephrine (3 μmol/L). Vasodilator concentration-response curves were constructed and compared before and after the addition of antagonists.

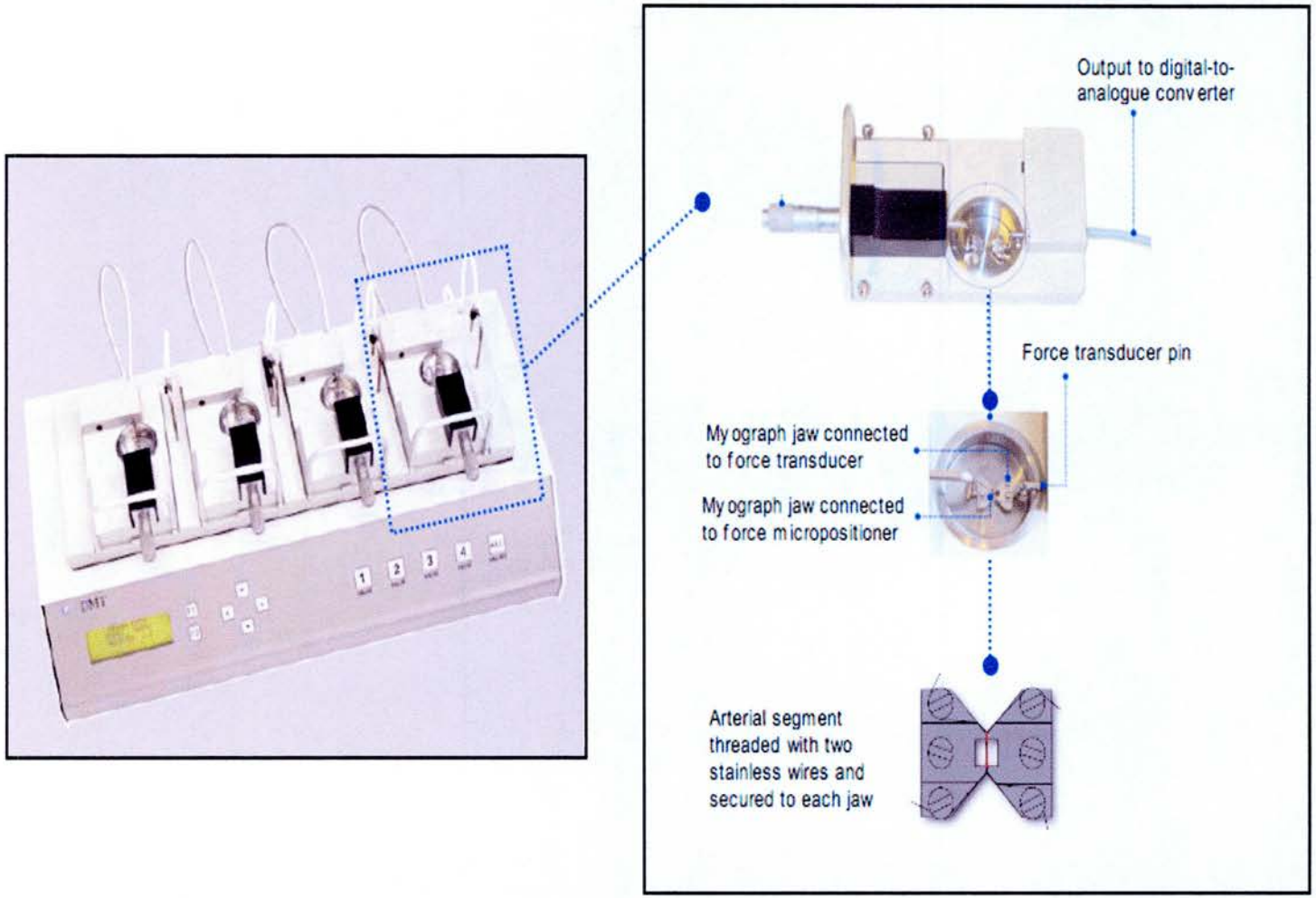


Figure 2.1 *Four chamber Mulvany-Halpern wire-myograph.*

2.4 IMMUNOHISTOCHEMISTRY

2.4.1 TISSUE PREPARATION

Freshly isolated artery segments were cryopreserved in optimal cutting temperature compound (Ted Pella, Inc, Redding, CA, USA) cooled by liquid nitrogen. Transverse 10 μm cryosections were prepared and mounted onto slides, air-dried, and stored at -20°C.

2.4.2 IMMUNOSTAINING

Before immunostaining, sections were fixed in acetone for 10 minutes at 4°C and rehydrated in Tris buffer solution, pH 7.6. One per cent hydrogen peroxide (H_2O_2) was added for 10 minutes to inhibit endogenous peroxidase and non-specific binding was blocked by incubation with 0.2% triton-X100 and 2% bovine serum albumin for 1 hour at room temperature. Immunostaining was performed with polyclonal rabbit antibodies against connexin 37, connexin 40 (1:50 dilution) or connexin 43 (1:100 dilution) at 4°C overnight (Zymed Laboratories, Inc., San Francisco, CA, USA). Negative control sections were incubated with non-immune goat IgG (SDS, Falkenberg, Sweden). Sections were subsequently washed in Tris buffer solution and incubated for 1 hour with biotinylated goat anti-rabbit IgG (1:300; Vector Laboratories, Burlingame, CA, USA). After rinsing with Tris buffer solution and application of 3,3-diaminobenzidine in H_2O_2 (DAB-Kit, Vector Laboratories), bound antibodies were treated with an avidin-biotin complex with peroxidase (Vectastain ABC Elite, Vector Laboratories) for 30 minutes. All slides were counterstained with

hematoxylin, dehydrated and mounted with Pertex (Histolab, Gothenburg, Sweden). Visualisation was achieved using conventional light microscopy.

2.5 ELECTROPHYSIOLOGIC OPTICAL MAPPING

2.5.1 TISSUE PREPARATION

Hearts were excised from male New Zealand white rabbits and placed in chilled Tyrode's solution (containing, [mmol/L]: Na 134.5, Mg 1.0, K 5.0, Ca 1.9, Cl 101.8, SO_4 1.0, H_2PO_4 0.7, HCO_3 20, acetate 20 and glucose 10). The left coronary artery was cannulated and perfused with oxygenated (95% O_2 and 5% CO_2) Tyrode's solution maintained at pH 7.4 and 37°C. Perfused left ventricular wedge preparations were dissected out and mounted in a custom built chamber which allowed access to the transmural surface for imaging. A transmural pseudo-electrocardiogram was recorded from two silver-silver chloride disc electrodes arranged across the ventricular wall. Two bipolar platinum stimulating electrodes were arranged directly opposite one another, at the border of the transmural surface with the endocardium and at the border of the transmural surface with the epicardium. The preparation was paced at 1.5 x diastolic threshold using a 2 ms pulse at a basic cycle length of 350 ms. Perfusion pressure and electrocardiogram were monitored throughout.

2.5.2 TRANSMURAL OPTICAL MAPPING

An optical mapping system was used to record transmural optical action potentials. Via a port in the perfusion cannula, the preparation was loaded with 100 μL of voltage sensitive dye (RH237, Molecular Probes, Eugene, OR, USA) dissolved in

dimethyl sulfoxide (DMSO; 1 mg/mL). 15 mmol/L 2,3-butanedione monoxime (Sigma-Aldrich, Dorset, UK) was used to eliminate motion artifact during optical recordings. Light from four 75 W tungsten-halogen lamps was passed through band-pass (525±25 nm) filters onto the transmural surface of the preparation. Emitted light was collected by the camera lens, long-pass filtered (>695 nm) and focused onto a charge-coupled device array (Redshirt Imaging, Decatur, GA, USA). Optical action potentials were recorded at 5 Hz from 676 sites.

2.5.3 Data analysis

Data analysis was performed using custom written software (Dr Francis Burton, University of Glasgow). A 2x2 Gaussian spatial filter was used and an automated analysis algorithm applied to all transmural optical action potentials (AP). A single investigator verified results visually. Activation time (AT) was defined as the midpoint between baseline and the peak of the action potential upstroke. Transmural conduction velocity was calculated using the activation time from the earliest activation on the edge of the stimulated transmural surface, to the earliest activation on the opposite side. Isochronal maps were constructed using algorithms written in Matlab software (MathWorks Inc., Natick, MA, USA).

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-gauge steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under 2% lignocaine (Xylocaine;

Astra Pharmaceuticals Ltd, Kings Langley, UK) local anaesthesia. The cannula was attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, UK) and patency maintained by infusion of saline (0.9%; Baxter Healthcare Ltd, Thetford, UK) via an IVAC P6000 syringe pump (Alaris Products, Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 mL/min.

2.6.2 Isolation of EDHF activity

As appropriate, cyclooxygenase activity was inhibited with a single 600 mg dose of oral aspirin (Dagra Pharma BV, Diemen, Netherlands) 1 hour prior to each study. This dose of aspirin rapidly inhibits bradykinin-stimulated endothelial production of PGI_2 by at least 85% with recovery developing over the next 6 hours [Heavey *et al*, 1985].

Nitric oxide production was inhibited with L-NMMA in the 'nitric oxide clamp'. After a 20-minute intra-arterial infusion of 0.9% saline, L-NMMA (8 μmol/min) was infused via the brachial artery. To compensate for L-NMMA induced basal vasoconstriction, forearm blood flow was restored to baseline using a titrated dose of exogenous NO in the form of intra-brachial sodium nitroprusside (90-900 ng/min). The titrated dose of sodium nitroprusside (SNP) was co-infused with L-NMMA throughout the study. This arrangement allows a constant 'clamped' delivery of exogenous NO whilst endogenous NO synthase activity is inhibited [Honing *et al*, 2000; Ueda *et al*, 2004].

Figure 2.2 *Forearm plethysmography set-up.*

2.6.3 INHIBITION OF PLATELET AGGREGATION DURING PAR-1 ACTIVATION

In studies including the intra-arterial administration of SFLLRN, the PAR-1 activating peptide, platelet aggregation was inhibited by the concurrent intra-arterial infusion of the platelet glycoprotein IIb/IIIa inhibitor, tirofiban (1250 ng/min; approximate local forearm concentration 50 ng/mL based upon estimated FBF of 25 mL/min). This dose of tirofiban inhibits SFLLRN-induced platelet aggregation without affecting platelet activation, FBF or the release of endogenous fibrinolytic factors [Guðmundsdóttir *et al*, 2006].

2.6.4 BLOOD FLOW MEASUREMENT

Blood flow was measured in the infused and non-infused forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges that were applied to the widest part of the forearm [Wilkinson and Webb, 2001]. During measurement periods, the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mmHg using E20 Rapid Cuff Inflators (D.E. Hokanson Inc, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mmHg pressure for 10 seconds in every 15 seconds to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 strain gauge plethysmograph (D.E. Hokanson Inc.) was processed by a PowerLab analogue-to-digital converter and Chart™ software (version 5.0.1, AD Instruments) and recorded onto a Dell Latitude laptop computer (Dell Computers Ltd, UK). Calibration was achieved using the internal standard of the plethysmograph.

2.6.5 SAMPLE SIZE CALCULATION

Based on power calculations derived from previous studies, a sample size of 8 gives 90% power of detection of a 22% change in FBF at a significance level of 5%. Colleagues have previously described the influence of a range of factors on blood flow in these vascular beds using sample sizes of 6-12 subjects [Newby *et al,* 1997; Newby *et al* 1998; Newby *et al,* 1999; Labinjoh *et al* 2001; Witherow *et al,* 2001; Cruden *et al,* 2005]. A smaller sample size was used in one instance in this thesis (Chapter 4, Protocol 1) in which tolerability and safety of ascending doses of a novel agent were assessed. The vasomotor effects of the top two doses used in that protocol were assessed further in a subsequent protocol with a greater sample size.

2.6.6 DATA ANALYSIS

Plethysmographic data were extracted from the Chart™ data files and FBF was calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Microsoft Excel for Mac 2004; Microsoft Corporation, USA). Recordings from the first 60 seconds after wrist cuff inflation were not used because of the variability in blood flow that this incurs [Benjamin *et al*, 1995]. Usually, the last five flow recordings in each 3-minute measurement period were calculated and averaged for each arm. The non-infused arm acted as a contemporaneous control for systemic effects and external influences upon FBF.

Analysis of all data collected during the forearm plethysmography study was undertaken by a single operator in a blinded fashion as appropriate. Forearm blood flow responses are reported as absolute blood flow responses (mL/100 mL tissue/min) in the infused and non-infused arm.

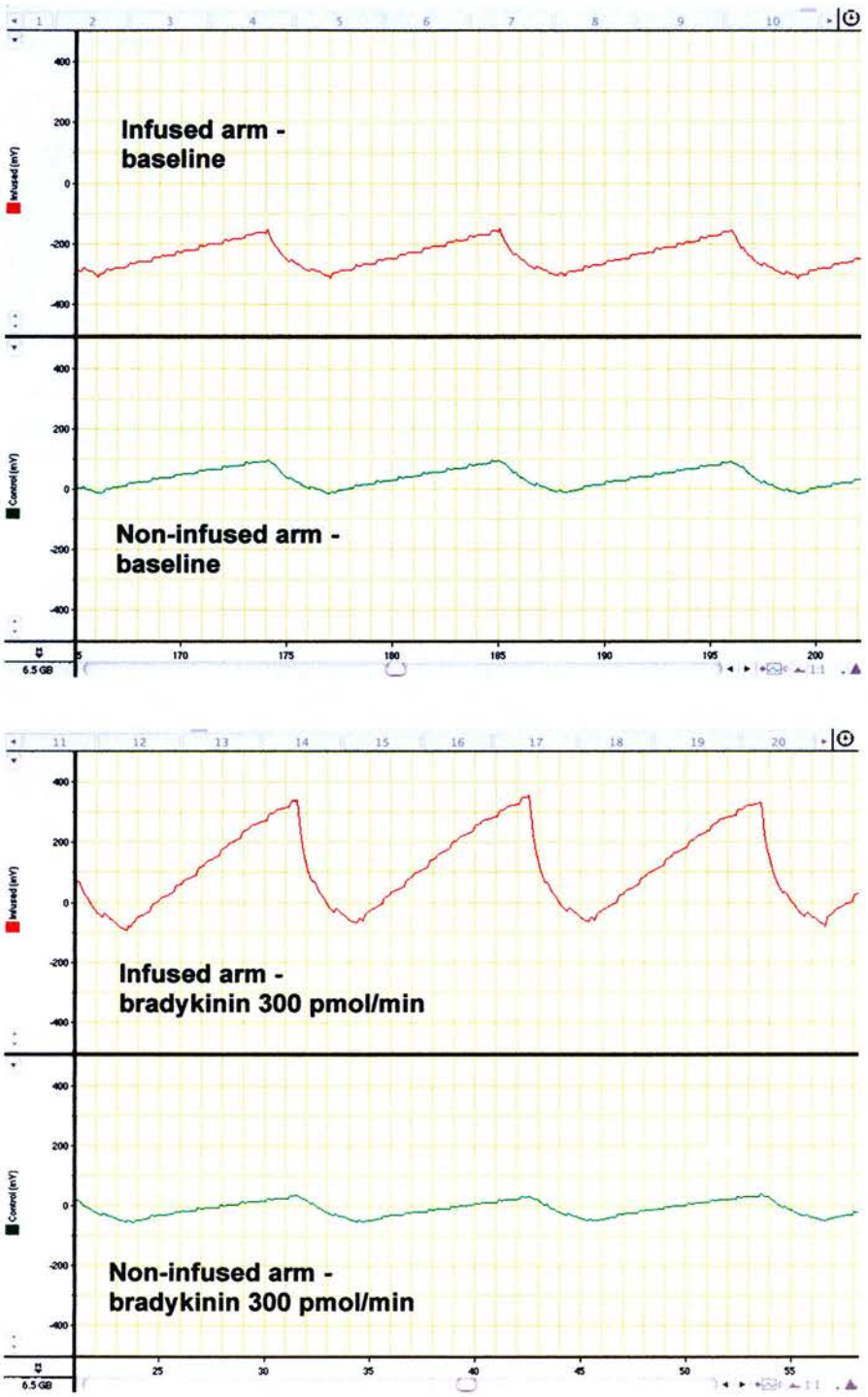


Figure 2.3 *Typical forearm plethysmographic traces.*

2.7 FIBRINOLYTIC AND HAEMOSTATIC PARAMETERS

2.7.1 FOREARM VENOUS SAMPLING

Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossa in both arms. Ten millilitres of blood were withdrawn simultaneously from each arm and collected into acidified buffered citrate (Stabilyte, Trinity Biotech Plc, Co. Wicklow, Ireland) and citrate (BD Vacutainer, BD UK Ltd, Oxford, UK) tubes, and kept on ice before being centrifuged at 2000 *g* for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay.

2.7.2 PLASMA HAEMOSTATIC AND FIBRINOLYTIC ASSAYS

Plasma t-PA antigen and activity (t-PA Combi Actibind Elisa Kit, Technoclone, Vienna, Austria) and PAI-1 antigen and activity (Elitest PAI-1 antigen and Zymutest PAI-1 Activity, Hyphen Biomed, Neuville-Sur-Oise, France) concentrations were determined by enzyme-linked immunosorbant assays. Intra-assay coefficients of variation were 7.0% and 5.5% for t-PA and PAI-1 antigen, and 4.0% and 2.4% for activity, respectively. Inter-assay coefficients of variability were 4.0%, 7.3%, 4.0% and 7.6% respectively. The sensitivities of the assays were 0.5 ng/mL, 2.5 ng/mL, 0.10 IU/mL and 5 AU/mL respectively. Von Willebrand factor antigen (Dako A/S, Glostrup, Denmark) and beta thromboglobulin antigen (Asserachrom Btg, Diagnostica Stago, France) were also determined using by enzyme-linked immunosorbent assays, both with sensitivities of 0.05 IU/mL. The intra-assay coefficients of variability were 5.2% and 5.4% respectively. The inter-assay coefficients of variation were 7.3% and 6.5% respectively.

2.7.3 HAEMATOCRIT MEASUREMENT

Haematocrit was determined using an automated Coulter counter (Beckman-Coulter ACt.8 Coulter Counter, High Wycombe, United Kingdom).

2.7.4 DATA ANALYSIS

Estimated net release of t-PA and PAI-1 activity and antigen was defined as the product of the infused FBF (based on the haematocrit [Hct] and the infused forearm blood flow [FBF]) and the concentration difference between the infused ([t-PA]Inf) and non-infused arms ([t-PA]Non-inf) [Oliver *et al*, 2005].

Estimated net forearm t-PA release = FBF x {1-Hct} x {[t-PA]Inf - [t-PA]Non-inf}

2.8 THE AELLIG DORSAL HAND VEIN TECHNIQUE

2.8.1 INTRAVENOUS ADMINISTRATION

A 23-gauge butterfly cannula (Hospira, Donegal Town, Ireland) was inserted into the dorsal hand vein in the direction of flow and attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, UK). Patency was maintained by infusion of saline via an IVAC P6000 syringe pump (Alaris Products, Basingstoke, UK). The total rate of intravenous infusions was maintained constant throughout all studies at 0.25 mL/min and the same hand vein was used for each study.

2.8.2 DORSAL HAND VEIN MEASUREMENT

The left hand was supported above the level of the heart by means of an arm rest. The internal diameter of the dorsal hand vein, distended by inflation of an upper arm cuff to 40 mmHg, was measured by the technique first decribed by Aellig [Aellig, 1981; Aellig, 1994a; Aellig, 1994b] using a linear variable differential transducer (LVDT; model 025 MHR, Lucas Schaevitz Inc). The LVDT was mounted on the dorsum of the hand using a small tripod, and a magnetised rod was passed through the core to rest on the summit of the vein approximately 1 cm proximal to the tip of the infusion cannula. Vertical displacement of the rod causes a linear change in the voltage generated by the LVDT reflecting changes in the internal diameter of the vein. The voltage output from the LVDT was transferred to a Macintosh Classic II computer (Apple Computer Inc, Cupertino, CA) using a MacLab analogue-to-digital converter (AD Instruments Ltd, Castle Hill, NSW, Australia) and Chart™ software (Version 5.4.2, AD Instruments).

2.8.3 SAMPLE SIZE CALCULATION

Based on power calculations from previous studies, a sample size of 6 gives 90% power to detect an 18% change in venous calibre at a significance level of 5% [Masumori *et al,* 1997; Sogo *et al,* 2000; Gudmundsdottir *et al,* 2006].

2.8.4 DATA ANALYSIS

Baseline vein diameter was calculated as the mean of the last three measurements during the initial infusion of saline, before the start of active drug infusion. In order to minimise the effects of any inter-subject and inter-study variability in hand vein

diameter, responses following infusion of agonists are expressed as percentage change in vein diameter from baseline [Haynes *et al*, 1994; Guðmundsdóttir *et al*, 2006].

2.9 STATISTICS

Data were examined by analysis of variance (ANOVA) with repeated measures and two-tailed paired Student's *t*-test as appropriate using GraphPad Prism (GraphPad Software, California, USA). All results are expressed as mean±standard error of the mean. Statistical significance was taken at the 5% level.

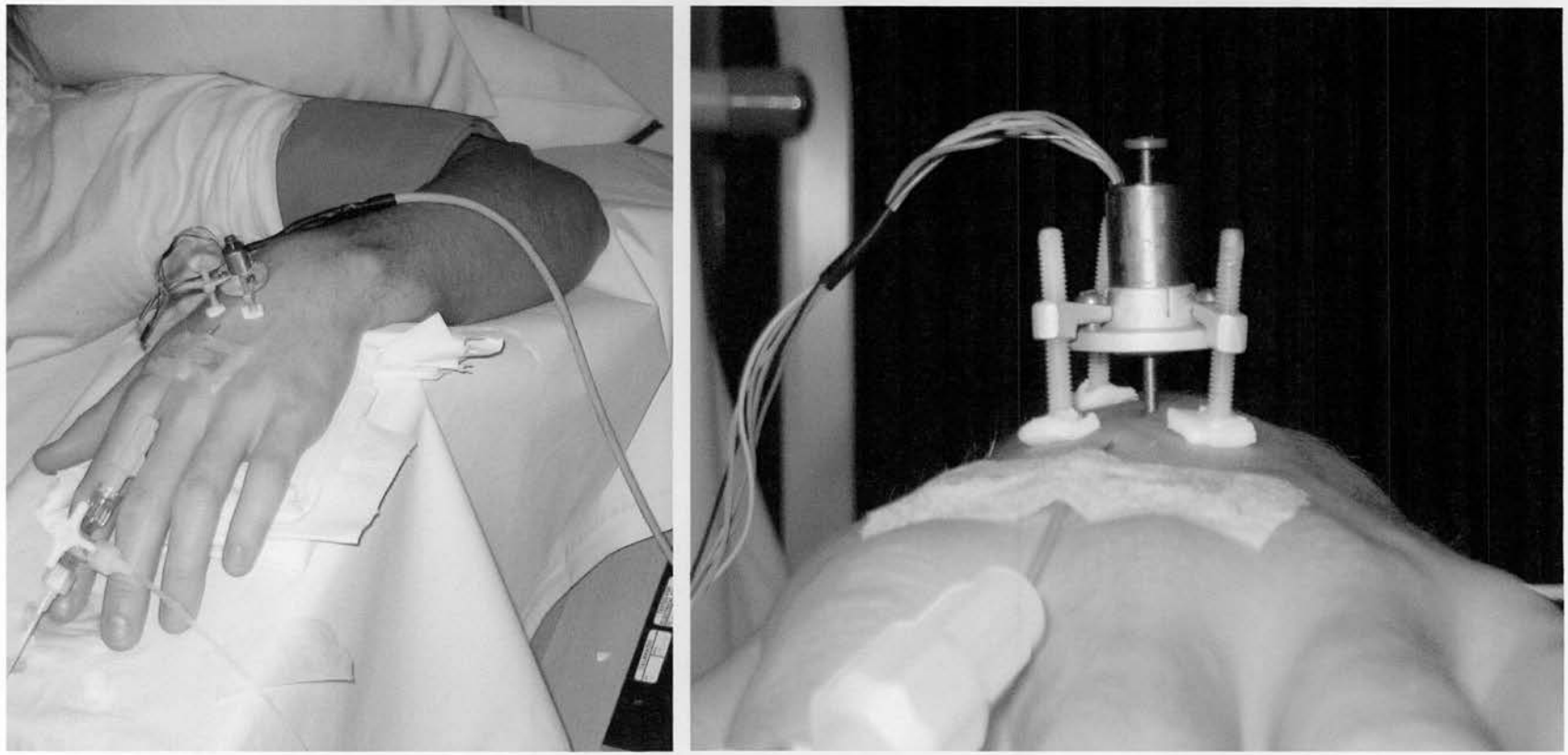


Figure 2.4 *Aellig dorsal hand vein technique.*

CHAPTER 3

CONNEXIN 43 MEDIATES ENDOTHELIUM-DERIVED HYPERPOLARISING FACTOR-INDUCED VASODILATATION IN SUBCUTANEOUS RESISTANCE ARTERIES FROM HEALTHY PREGNANT WOMEN

Lang NN, Luksha L, Newby DE, Kublickiene K.
Connexin 43 Mediates Endothelium-Derived Hyperpolarising Factor-Induced Vasodilatation in Subcutaneous Resistance Arteries from Healthy Pregnant Women.
Am J Physiol Heart Circ Physiol 2007;**292**(2):H1026-1032.

3.1 SUMMARY

This study assessed the role of gap junctions in EDHF-mediated relaxation of human arteries using CMPs designated ^{37,43}Gap27, ^{40}Gap27 and ^{43}Gap26 according to homology with the major vascular connexins (Cx37, 40 and 43). Resistance arteries were obtained from subcutaneous fat biopsies of healthy pregnant women undergoing elective caesarean section. Endothelium-dependent vasodilatation to bradykinin was assessed using wire-myography. N^{ω}-nitro-L-arginine-methyl ester and indomethacin (NO synthase and cyclooxygenase inhibitors respectively) attenuated maximal relaxation to bradykinin (R_{max}) by ~50%. Co-incubation with L-NAME, indomethacin and the combined CMPs (^{37,43}Gap27, ^{40}Gap27 and ^{43}Gap26) almost abolished relaxation to bradykinin (R_{max} 12.2±3.7%). In arteries incubated with L-NAME and indomethacin, the addition of either ^{37,43}Gap27 or ^{40}Gap27 had no significant effect on R_{max} whilst ^{43}Gap26 caused marked inhibition (R_{max} 21±6.4%, P=0.005 *vs.* L-NAME plus indomethacin alone) that was similar to the triple combination. Endothelium-independent vasorelaxation was unaffected by CMPs, L-NAME or indomethacin. Immunohistochemistry demonstrated connexin 37, 40 and 43 expression in the endothelium and vascular smooth muscle. These results demonstrate that, in pregnant women, EDHF-mediated vasorelaxation of subcutaneous resistance arteries is dependent on connexin 43 and gap junctions.

3.2 INTRODUCTION

Nitric oxide, the original *endothelium-derived relaxing factor,* and PGI_2 have now been well characterised. The elucidation of their roles in vascular physiology and pathophysiology has been fundamental to recent advances in the treatment and prevention of many cardiovascular diseases.

Evidence points to the existence of a third powerful vasodilator called EDHF [Busse *et al*, 2002; Griffith, 2004; Sandow, 2004]. Endothelium-derived hyperpolarising factor's contribution to endothelium-dependent vasorelaxation is greatest in small resistance arteries [Berman et al, 2002] and, as such, is ideally suited to the control of systemic blood pressure and local tissue perfusion. As well as its involvement in normal physiological responses, EDHF is believed to play an important role in vascular pathophysiology. It has been suggested to act as a 'back-up' mechanism in conditions associated with decreased NO bioavailability, such as heart failure [Malmsjo *et al*, 1999; Katz and Krum, 2001]. Furthermore, failure of EDHF to compensate in this way has been implicated in the pathogenesis of pre-eclampsia [Kenny *et al*, 2002].

Despite intensive research, the exact nature of EDHF and its mechanism of action remain unclear. This lack of understanding has precluded its manipulation as a therapeutic target [Feletou and Vanhoutte, 2004]. Multiple candidates have been proposed but none have been confirmed as a unifying agent constituting EDHF. As

such, the term *endothelium-derived hyperpolarising factor* may be misleading and EDHF may represent a mechanism rather than a specific factor *per se*.

The case for gap junctions as a central component of the EDHF mechanism has strengthened [Sandow *et al*, 2002; Griffith, 2004]. These aqueous pores facilitate the transfer of either small hydrophilic molecules or ionic charge for the conduction of hyperpolarisation from the endothelium to smooth muscle and from smooth muscle cell to smooth muscle cell. Gap junctions are composed of two hemichannels comprising six connexin subunits each. Cx37, Cx40 and Cx43 are particularly associated with mammalian endothelium and vascular smooth muscle [Griffith, 2004].

Diverse agents have been employed as putative gap junction blockers but their other non-specific, EDHF-independent effects have limited their use. In contrast, the CMPs are highly selective and specific [Griffith, 2004; Matchkov *et al*, 2004]. These short synthetic peptides correspond to conserved amino acid sequences in the first (Gap 26) and second (Gap 27) extracellular loops of Cxs37, 40 and 43 and cause disruption of connexin function [Chaytor *et al*, 1999].

Pregnancy is associated with a decrease in peripheral vascular resistance and enhanced endothelium-dependent vasodilatation [Gillham *et al*, 2003]. It has previously been reported that EDHF accounts for approximately 50% of endothelium-dependent vasodilatation in these arteries. Furthermore, using the putative gap junction blocker 18α-glycyrrhetinic acid, a potential role of gap

junctions was identified in EDHF responses [Luksha et al, 2004]. In this study, we tested the hypothesis that gap junction blockade with CMPs inhibits EDHF-mediated vasorelaxation in resistance vessels from pregnant women. Furthermore, we examined the expression of the three main vascular connexin subtypes in these vessels. Using CMPs, the current study represents the first assessment of the functional role of gap junctions and their connexin components in EDHF-mediated relaxation in the human vasculature.

3.3 MATERIALS AND METHODS

3.3.1 BIOPSY COLLECTION AND VESSEL PREPARATION

The investigation was undertaken with the approval of the local Research Ethics Committee, with the written informed consent of each patient, and in accordance with the principles outlined in the Declaration of Helsinki.

Subcutaneous fat biopsies were obtained from healthy women undergoing elective caesarean section. Women with a history of diabetes mellitus, hypertension or any other significant past medical history were excluded from participation. Women with abnormal renal or hepatic function were also excluded. Biopsies were taken from the incision margin and immediately placed in cold PSS (NaCl 119 mmol/L, KCl 4.7 mmol/L, $CaCl_2$ 2.5 mmol/L, $MgSO_4$ 1.17 mmol/L, $NaHCO_3$ 25 mmol/L, KH_2PO_4 1.18 mmol/L, EDTA 0.026 mmol/L, glucose 5.5 mmol/L).

Resistance arteries were dissected free of surrounding fat and connective tissue, and

cut into segments approximately 2 mm long. All arteries were kept in PSS at 4°C and used on the day of biopsy. Arteries were mounted in the organ baths of a four-channel wire-myograph (multimyograph, model 610, Danish Myo Technology; Aarhus, Denmark) as described previously [Luksha *et al*, 2004]. Following a 30-minute equilibration period, a passive circumference-tension curve was created for each segment in order to set optimum resting tension, as described previously [Mulvany and Halpern, 1976; Luksha *et al*, 2004]. This semi-automated procedure also allows the calculation of arterial diameter. Calibration and data processing were performed using Myodac software (version 2.1, Danish Myo Technology) on a personal computer. All solutions were refreshed every 30 minutes.

Constriction was elicited using norepinephrine 3 μmol/L. Endothelium-dependent vasodilatation was assessed by the addition of a single dose of bradykinin (1 μmol/L) to each chamber. Arteries that did not achieve at least 60% vasorelaxation to bradykinin were excluded from the study.

3.3.2 Experimental protocols

Arteries were incubated for 40 minutes in either PSS alone or with the NO synthase inhibitor L-NAME; 300 μmol/L, and the cyclooxygenase inhibitor indomethacin (10 μmol/L), to block the production of NO and PGI_2 respectively. Arteries were then preconstricted with norepinephrine (3 μmol/L) and the concentration-response curve for bradykinin (0.001–3 μmol/L) assessed. Arteries then underwent 90 minutes incubation with CMPs, with or without L-NAME and indomethacin as detailed below. The concentration-response curve to bradykinin was then reassessed. At the

end of each experiment, endothelium-independent vasorelaxation was assessed in response to sodium nitroprusside (0.1 mmol/L). The doses of the CMPs used were based upon prior published work [Chaytor *et al*, 2003; Griffith, 2004].

3.3.3 Gap junction inhibition with and without NO synthase/cyclooxygenase inhibition

Arteries initially incubated in PSS alone were incubated with PSS plus the combined CMPs ^{37,43}Gap27 (300 μmol/L), ^{40}Gap27 (300 μmol/L) and ^{43}Gap26 (300 μmol/L). The contribution of gap junctions to EDHF-mediated vasorelaxation with concurrent NO synthase/cyclooxygenase inhibition was assessed by pre-incubating with L-NAME and indomethacin before administering the combined CMPs (as above) in the continued presence of L-NAME and indomethacin.

3.3.4 Contribution of connexin subtypes to EDHF-mediated relaxation

Arteries pre-incubated with L-NAME and indomethacin alone were incubated with L-NAME and indomethacin plus either i) ^{37,43}Gap27 plus ^{40}Gap27 (450 μmol/L each); ii) ^{37,43}Gap27 (900 μmol/L); iii) ^{40}Gap27 (900 μmol/L); or iv) ^{43}Gap26 (900 μmol/L). Total peptide concentration in each experiment was kept constant at 900 μmol/L.

3.3.5 The effects of ^{43}Gap26 alone on endothelium-dependent and independent vasorelaxation

Arteries initially incubated in PSS alone were incubated with ^{43}Gap26 (900 μmol/L) for 90 minutes. A concentration-response curve to bradykinin was constructed (as

above), before and after ^{43}Gap26. In further experiments, a concentration-response curve was constructed for either sodium nitroprusside (0.001-1 μmol/L) or the endothelium-independent, ATP-sensitive potassium channel opener, pinacidil (0.001-1 μmol/L) before and after incubation with ^{43}Gap26 (again without L-NAME and indomethacin).

3.3.6 Immunohistochemistry

Freshly isolated artery segments were cryopreserved in optimal cutting temperature compound cooled by liquid nitrogen. Transverse 10 μm cryosections were prepared and mounted onto slides, air-dried, and stored at -20°C. Sections were immunostained with polyclonal rabbit antibodies against mouse connexins (Cx37 and Cx40 (1:50 dilution) or Cx43 (1:100 dilution) at 4°C overnight (Zymed Laboratories, Inc., San Francisco, CA, USA) [Li *et al*, 1998; Nakamura *et al*, 1999]. Negative control sections were incubated with non-immune goat IgG (SDS, Falkenberg, Sweden). All slides were counterstained with hematoxylin, dehydrated and mounted with Pertex (Histolab, Gothenburg, Sweden).

3.3.7 Drugs and chemicals

All drugs and chemicals except the CMPs and antibodies were obtained from Sigma (St. Louis, MO). The CMPs, ^{37,43}Gap27 (SRPTEKTIFII), ^{40}Gap27 (SRPTEKNVFIV) and ^{43}Gap26 (VCYDKSFPISHVR) were purchased from American Peptide Company, Inc. (Sunnyvale, CA). All drugs except indomethacin and L-NAME were dissolved in PSS before every experiment. Indomethacin was dissolved in pure ethanol. L-NAME was dissolved in distilled water.

3.3.8 DATA ANALYSIS

All data are expressed as mean±SEM. Maximum vasorelaxation (R_{max}) is expressed as percentage return to basal tone produced by the highest concentration of badykinin (3 μmol/L) after norepinephrine preconstriction. Analysis of variance was used to compare paired bradykinin concentration-response curves before and after incubation with CMPs and between groups. Bonferroni's correction for multiple comparisons was used for the analysis of pooled data. Paired Student's *t*-test was used to compare norepinephrine preconstriction before and after incubation with different agents (GraphPad Prism v4.0). Statistical significance was taken at the 5% level.

3.4 RESULTS

Subcutaneous fat biopsy specimens were obtained from 14 pregnant women (5 nulliparous) with a median age of 27 years (range 25-42) and a median gestational age of 38 weeks (range 37-40) undergoing planned caesarean section due to breach presentation (n=4), previous caesarean section (n=8) and for psychological reasons (n=2).

A total of 31 arteries dissected from 14 biopsies were included in the experiment. Mean internal diameter of artery segments used was 221±8 μm. Vessels with an internal diameter of 100-300 μm were considered to represent resistance arteries, in line with previous definitions [Christensen and Mulvany, 2001]. There were no

significant differences between artery segments used in each protocol with respect to internal diameter and initial constrictor response to norepinephrine (data not shown).

3.4.1 CONTRIBUTION OF EDHF TO BRADYKININ-MEDIATED VASORELAXATION

Compared with all arteries incubated in PSS alone (n=18), incubation with L-NAME and indomethacin (n=27) reduced maximal vasorelaxation to bradykinin by approximately 50% (R_{max} 90.3±2.2% PSS alone *vs.* 48.1±2.6% L-NAME/indomethacin, P<0.0001). Pilot studies confirmed that bradykinin concentrations of more than 3 μmol/L failed to further relax vessels incubated in PSS alone.

Gap junction inhibition with the combined CMPs (^{37,43}Gap27 plus ^{40}Gap27 plus ^{43}Gap26 [300 μmol/L each]) substantially reduced maximal relaxation to bradykinin (R_{max} 46.6±2.8% combined CMPs *vs.* 89.4±3.5% PSS, P=0.023 [n=6]). The inhibition of gap junction mediated communication with combined CMPs decreased maximal bradykinin-induced vasorelaxation to a similar extent as L-NAME plus indomethacin alone (Figure 3.1).

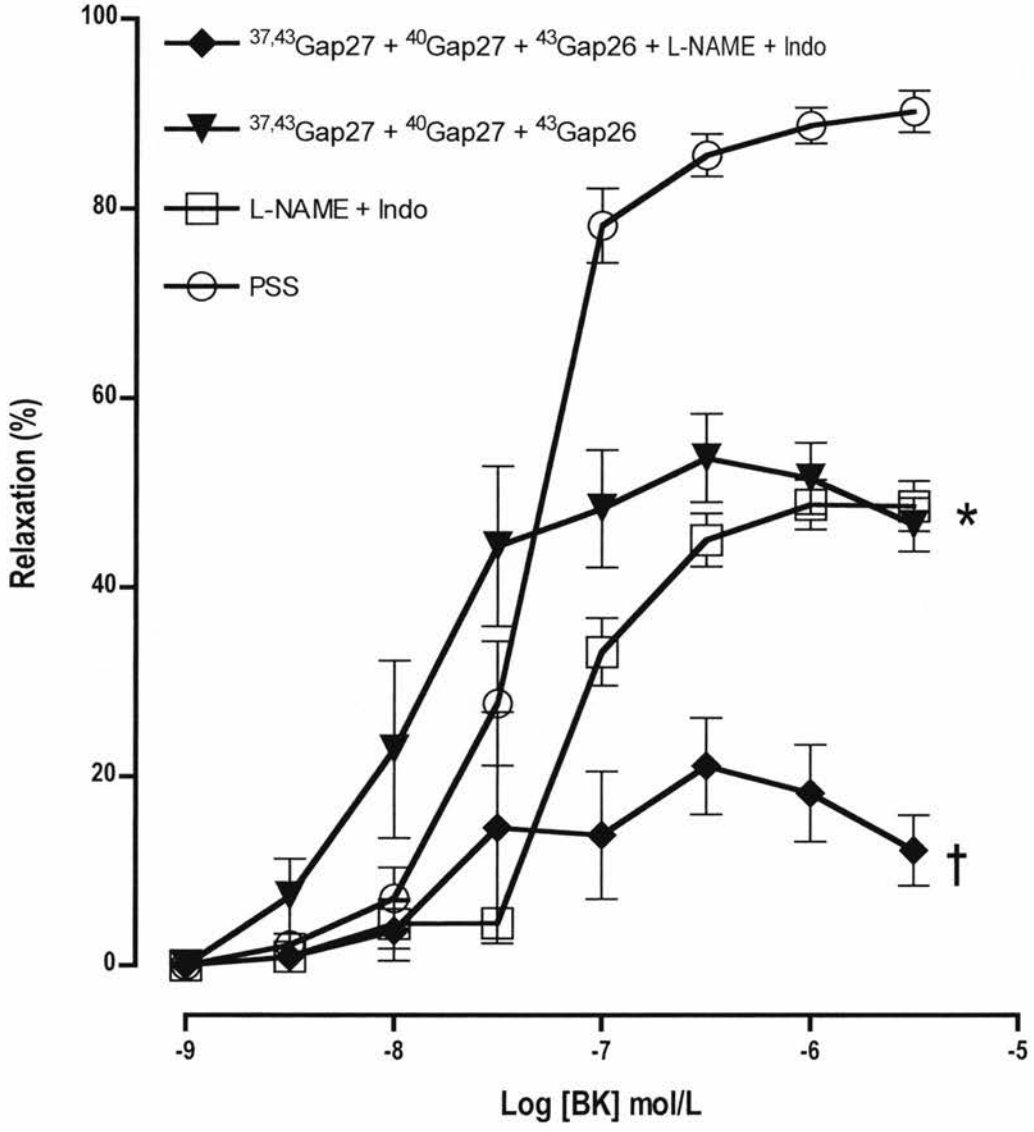


Figure 3.1 *Concentration response curves for bradykinin (BK) in subcutaneous arteries incubated in physiological salt solution (PSS) (n=18), L-NAME plus indomethacin (Indo) (n=25) or the combined connexin mimetic peptides (^{37,43}Gap27, ^{40}Gap27 and ^{43}Gap26 at 300 µmol/L each) with or without L-NAME plus indomethacin (n=6 for both).*
**P<0.001 vs. PSS; †P<0.001 vs. L-NAME and indomethacin alone.*

Combined NO synthase, cyclooxygenase and gap junction inhibition with the combined CMPs plus L-NAME and indomethacin almost abolished the vasorelaxation to bradykinin (R_{max} 12.2±3.7% combined CMPs plus L-NAME and indomethacin *vs.* 49.0±5.1% L-NAME and indomethacin, P=0.0004 [n=6]; Figure 3.1).

3.4.2 Contribution of different connexin subtypes to EDHF-mediated vasorelaxation

Incubation with ^{37,43}Gap27 (450 μmol/L) plus ^{40}Gap27 (450 μmol/L) in addition to L-NAME and indomethacin did not reduce maximal vasorelaxation to bradykinin when compared with the same vessels incubated in L-NAME and indomethacin alone (R_{max} 38.2±5.6% L-NAME and indomethacin plus ^{37,43}Gap27, ^{40}Gap27 *vs.* 40.6±6.1% L-NAME plus indomethacin, P=0.319 [n=5]; Figure 3.2).

The addition of ^{37,43}Gap27 (900 μmol/L) or ^{40}Gap27 (900 μmol/L) to L-NAME and indomethacin had no effect on maximal bradykinin-induced vasorelaxation compared to the same arteries incubated with L-NAME and indomethacin alone (R_{max} 43.6±1.4% ^{37,43}Gap27 with L-NAME plus indomethacin *vs.* 49.6±4.87% L-NAME plus indomethacin alone, P=0.39 [n = 5]; R_{max} 46.3±5.5% ^{40}Gap27 with L-NAME plus indomethacin *vs.* 45.0 ± 2.2% L-NAME plus indomethacin alone, P=0.37, [n=4]; Figure 3.2). However, the inhibition of connexin 43 with ^{43}Gap26 (900 μmol/L) in combination with L-NAME plus indomethacin resulted in a marked decrease in maximal vasorelaxation to bradykinin when compared to the same arteries incubated with L-NAME plus indomethacin alone (R_{max} 21.0±6.4% for

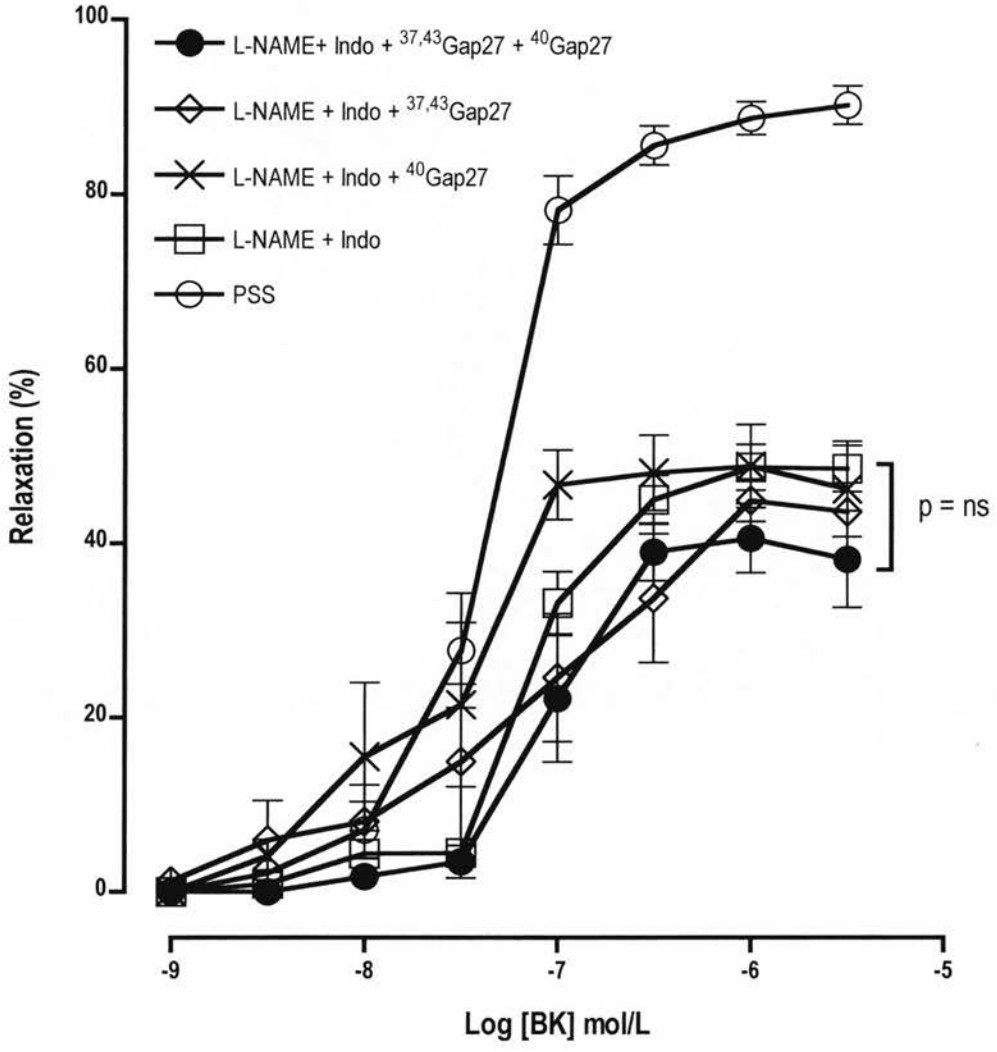


Figure 3.2 *Concentration response curves for bradykinin (BK) in subcutaneous arteries incubated in physiological salt solution (PSS) (n=18), L-NAME and indomethacin (Indo) alone (n=25) or with L-NAME and indomethacin plus: a) ^{37,43}Gap27 and ^{40}Gap27 (450 µmol/L each; n=5), b) ^{37,43}Gap27 (900 µmol/L; n=5), or c) ^{40}Gap27 (900 µmol/L; n=4).*
P>0.05 for L-NAME and indomethacin alone vs. L-NAME and indomethacin in combination with ^{37,43}Gap27 and/or ^{40}Gap27.

^{43}Gap26 with L-NAME plus indomethacin *vs.* 52.45±8.3% for L-NAME plus indomethacin alone, P=0.0047 [n=5]). Maximal bradykinin-induced relaxation after incubation with L-NAME plus indomethacin with ^{43}Gap26 (900 μmol/L) was not different from maximal relaxation after incubation with the three combined CMPs (each at 300 μmol/L) (unpaired data, P=0.397; Figure 3.3).

3.4.3 THE EFFECTS OF ^{43}GAP26 ALONE ON ENDOTHELIUM-DEPENDENT VASORELAXATION

Incubation with ^{43}Gap26 (900 μmol/L) (without L-NAME and indomethacin) reduced maximal bradykinin-induced relaxation when compared with the same arteries incubated in PSS alone (R_{max} 40.6±15.8% for ^{43}Gap26 alone *vs.* 85.8±5.5% for PSS; P=0.039 [n=5]). Maximal bradykinin-induced relaxation after incubation with ^{43}Gap26 (900 μmol/L) alone was not significantly different from that observed after incubation with the three combined CMPs (each at 300 μmol/L) in PSS (R_{max} 40.6±15.8% for ^{43}Gap26 alone *vs.* 46.6±2.8% combined CMPs in PSS; P=0.69)

3.4.4 ENDOTHELIUM-INDEPENDENT VASORELAXATION AND VASOCONSTRICTION

Relaxation evoked by the exogenous NO donor, sodium nitroprusside (SNP; 0.1 mmol/L) were unaffected throughout all studies (data not shown). Norepinephrine-induced vasoconstriction of 2.1±0.07 mN/mm^2 and was unaffected by any of the incubation protocols. ^{43}Gap26 alone had no effect upon responses to the endothelium-independent vasodilators, sodium nitroprusside and pinacidil (n=5 for both) (Figure 3.4).

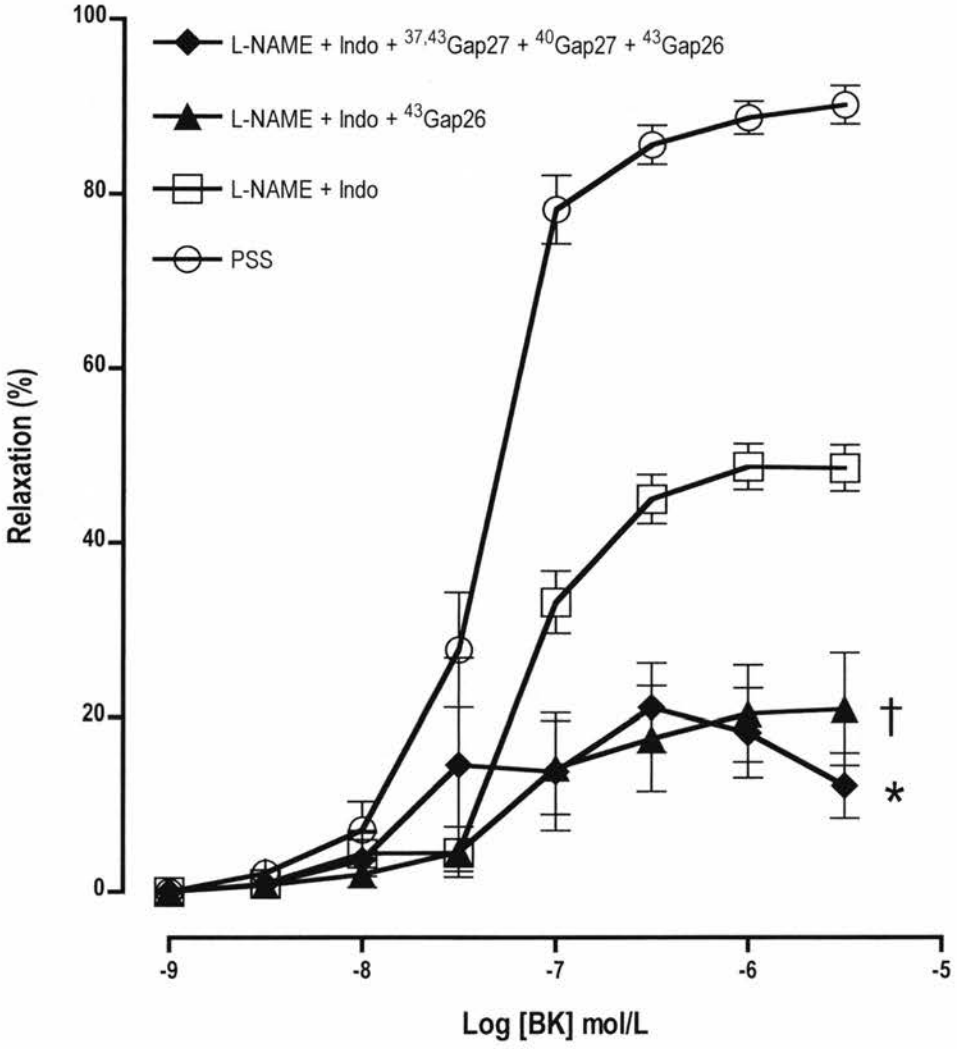


Figure 3.3 *Concentration response curves for bradykinin (BK) in subcutaneous arteries incubated in physiologicial salt solution (PSS) (n=18), L-NAME and indomethacin (Indo) alone (n=25) or with L-NAME and indomethacin plus: a) ^{37,43}Gap27, ^{40}Gap27 and ^{43}Gap26 (300 µmol/L each; n=6), or b) ^{43}Gap26 (900 µmol/L; n=5).*
**P<0.001 vs. L-NAME and indomethacin; †P<0.005 vs. L-NAME and indomethacin.*

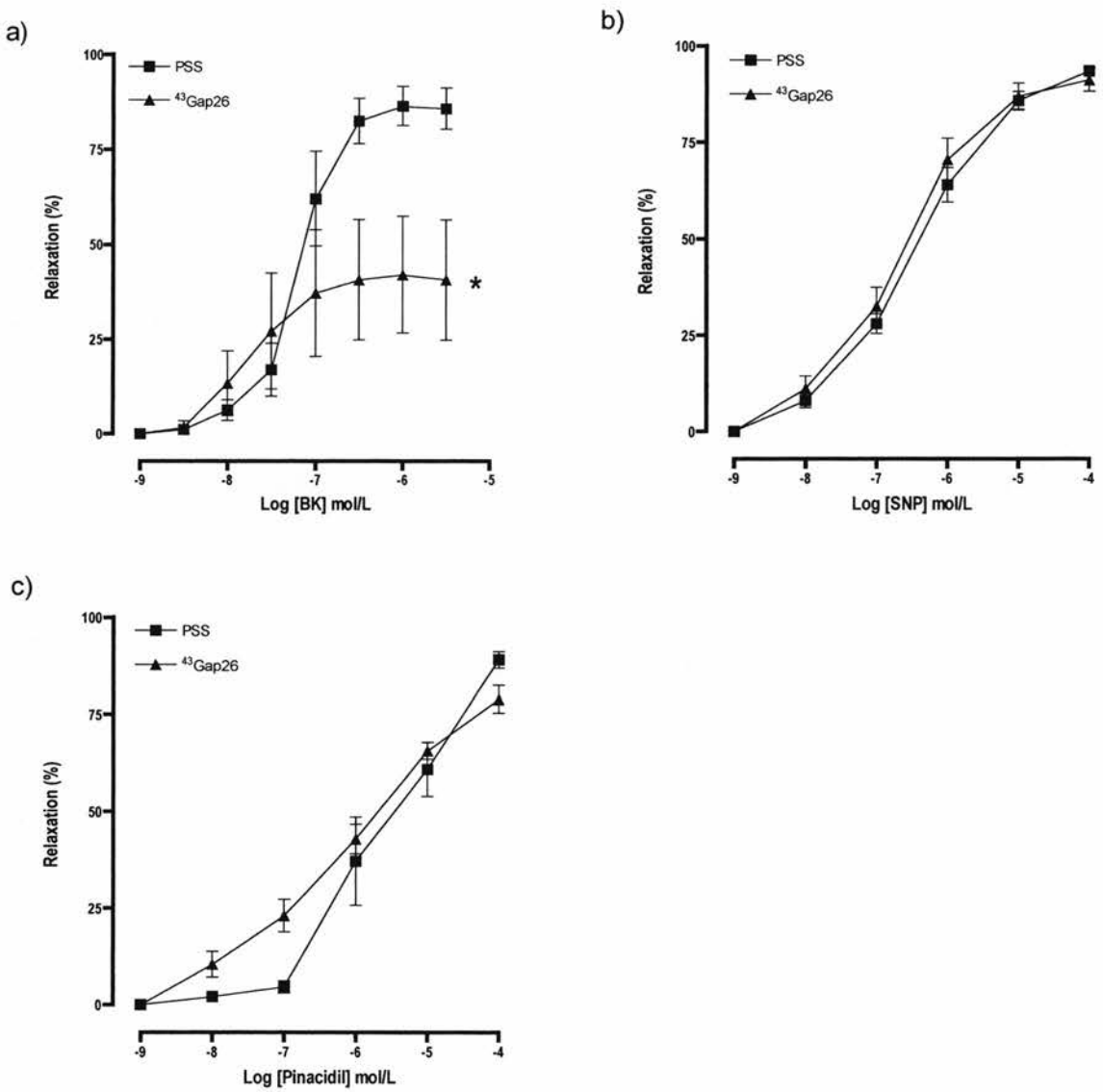


Figure 3.4 *Concentration response curves for a) bradykinin (BK), b) sodium nitroprusside (SNP) and c) pinacidil in subcutaneous arteries incubated in physiological salt solution (PSS) and ^{43}Gap26 (900 µmol/L) (n=5 for each).*
**P<0.05 vs. PSS.*

3.4.5 IMMUNOHISTOCHEMISTRY

Immunohistochemistry demonstrated the consistent presence of Cx37, 40 and 43 in arterial segments (Figure 3.5). Connexins 37, 40 and 43 were seen in both vascular smooth muscle and endothelial cells although Cx40 was predominantly found in the endothelium.

3.5 DISCUSSION

We have made the first assessment of the role of gap junctions and connexin subtypes in EDHF-mediated vasorelaxation of human arteries using highly specific gap junction inhibitors. We have shown that EDHF-mediated vasorelaxation relies upon gap junction communication acting principally through the connexin 43 subtype.

We have confirmed that subcutaneous resistance arteries obtained from pregnant women retain approximately 50% of their endothelium-dependent relaxant capacity after the inhibition of endothelial NO and PGI_2 synthesis with L-NAME and indomethacin respectively. This L-NAME and indomethacin insensitive component of endothelium-dependent vasorelaxation is widely regarded to reflect EDHF activity. Furthermore, incubation with a combination of ^{37,43}Gap27, ^{40}Gap27 and ^{43}Gap26 (300 μmol/L each) attenuated vasorelaxation to bradykinin to a similar extent as L-NAME and indomethacin alone and revealed that gap junctions are required for approximately 50% of bradykinin- induced vasorelaxation.

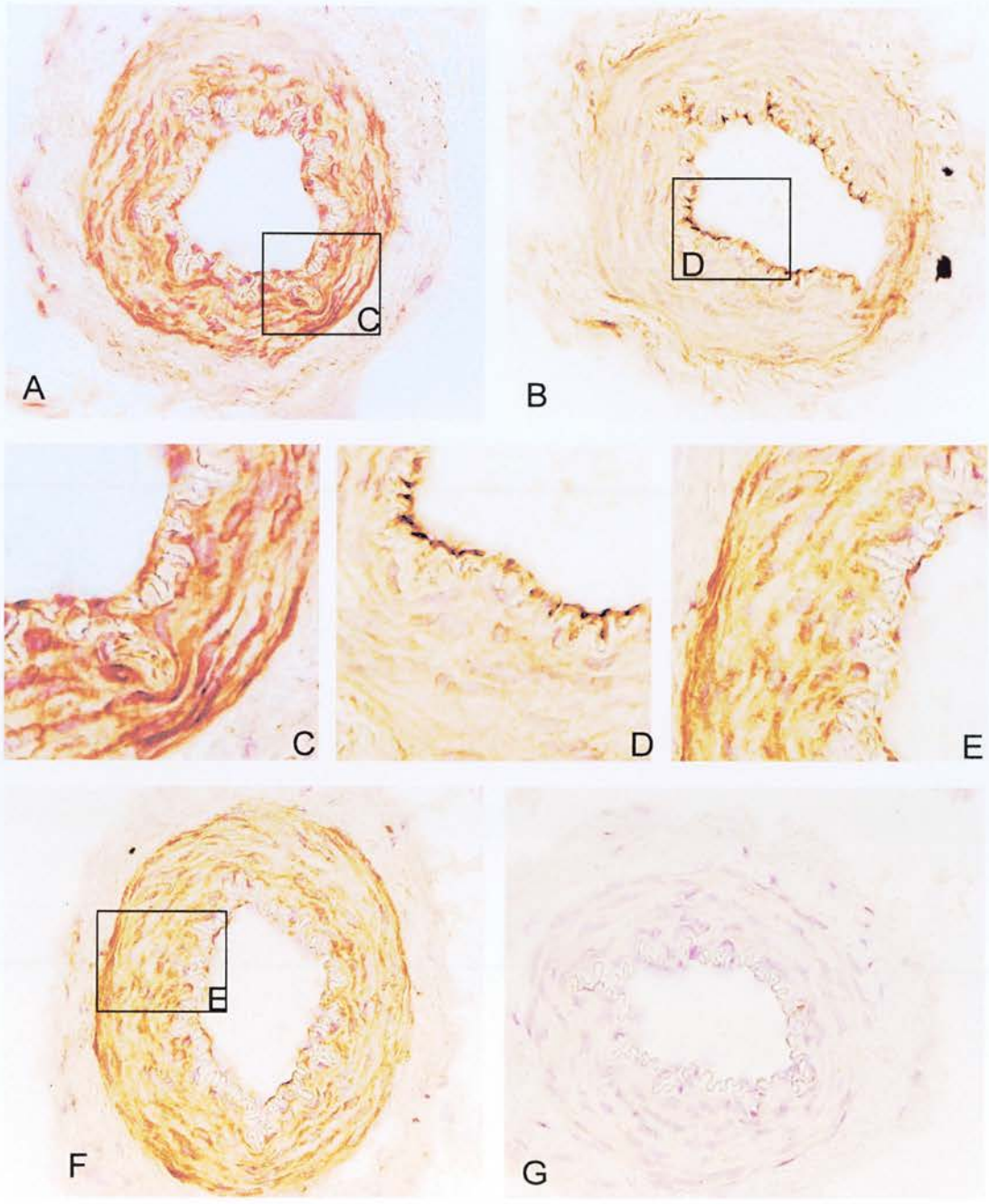


Figure 3.5 *Immunohistochemistry of pregnant human subcutaneous resistance arteries.* ***A, C:*** *Staining for Cx43 seen in both smooth muscle and endothelial cell layers.* ***B, D:*** *Staining for Cx40 principally seen in the endothelium.* ***E, F:*** *Staining for Cx37 seen in endothelium and smooth muscle.* ***G:*** *control images of arteries incubated without primary antibodies show no staining for connexins. All images x40.*

Co-incubation of the triple CMP combination with L-NAME and indomethacin virtually abolished bradykinin-induced vasorelaxation. That is, the L-NAME/indomethacin insensitive component of bradykinin-induced relaxation was inhibited by gap junction blockade. This provides further evidence of a pivotal role for gap junctions in EDHF-mediated relaxation in these vessels. These findings are in keeping with a previous study in similar vessels [Luksha *et al*, 2004] where bradykinin-mediated relaxation was abolished by a combination of L-NAME, indomethacin and 18α-glycyrrhetinic acid.

We went on to assess the effects of each of the three CMPs in order to infer the relative functional importance of Cxs37, 40 and 43. ^{43}Gap26 exerted marked inhibition of EDHF-mediated relaxation to bradykinin. Indeed, at 900 μmol/L, its inhibitory effect did not differ from that seen after incubation with the triple combination of CMPs. The marked inhibition of vasodilatation achieved by incubation with ^{43}Gap26 contrasts with the lack of inhibition seen after incubation with ^{37,43}Gap27 and ^{40}Gap27 in combination (450 μmol/L each) and individually (900 μmol/L). Furthermore, in the absence of L-NAME and indomethacin, ^{43}Gap26 caused a significant attenuation in maximal vasodilatation to bradykinin. Taken together, this suggests that the combined inhibitory effect of all three CMPs is derived predominantly from ^{43}Gap26 activity and implicates Cx43 as the dominant connexin isoform involved in the mediation of EDHF responses in these vessels.

Data from combined eNOS/cyclooxygenase knockout mice imply a greater role for EDHF in females than in males [Scotland *et al*, 2005] whilst other animal models

have demonstrated dynamic Cx43 expression under the influence of oestrogen. Pregnant rats exhibit enhanced EDHF-mediated vasodilatation and up-regulation of vascular Cx43 expression [Dantas *et al*, 1999]. These effects are markedly diminished in ovariectomised animals but can be restored by exogenous oestrogen supplementation [Liu *et al*, 2002]. Furthermore, an increase in Cx43 expression in response to increased shear stress and mechanical load has been demonstrated [Rummery and Hill, 2004]. The rise in circulating blood volume associated with normal human pregnancy could invoke similar changes in Cx43 expression and function and account for the predominant functional importance of Cx43 in our study. Although diversity exists between species and the sexes, EDHF makes a large contribution to endothelium-dependent vasodilatation in various vascular beds in men and non-pregnant women [Nakashima *et al*, 1993; Ohlmann *et al*, 1997; Urakami-Harasawa *et al*, 1997; Wallerstedt and Bodelsson, 1997; Buus *et al*, 2000; Coats *et al*, 2001; McIntyre *et al*, 2001]. The present study supports a clear role for Cx43 in vascular responses of pregnant women [Dantas *et al*, 1999]. Whether or not Cx43 is of such fundamental importance in men, non-pregnant women and in other vascular beds remains to be established.

The interpretation of results using older putative gap junction blockers has been limited by their non-specific effects. Indeed, 18α- glycyrrhetinic acid can affect ion channel conduction to attenuate endothelial cell hyperpolarisation directly. Confidence with the CMPs growing: they do not suppress endothelial hyperpolarisation directly and do not exert non-gap junctional effects [Griffith, 2004; Matchkov *et al*, 2006]. Their exact mechanism of action remains unclear but they are

thought to interfere with connexin gating properties. Unlike 18α- glycyrrhetinic acid, they do not affect the structural integrity, number or distribution of connexins [Berman *et al*, 2002]. When administered in combination and individually in this study, the CMPs did not have any influence upon vasorelaxation to sodium nitroprusside or preconstriction to norepinephrine, suggesting that they specifically inhibit endothelium-dependent vasorelaxation without interfering with vascular smooth muscle function. Whilst ^{43}Gap26 alone significantly attenuates maximal relaxation to bradykinin, it does not have any influence upon the concentration-response curve obtained after exposure to the endothelium-independent vasodilators, sodium nitroprusside and pinacidil. This provides more robust evidence that this CMP does not exert non-specific or toxic effects upon smooth muscle function.

If Cx43 has a dominant role then one might expect EDHF-mediated vasorelaxation to be inhibited by ^{37,43}Gap27 as well as by ^{43}Gap26. ^{37,43}Gap27 shares sequence homology with the Gap27 domain of both connexin 37 and 43 whereas ^{43}Gap26 shares homology with the Gap26 domain of Cx43 only. Both ^{37,43}Gap27 and ^{43}Gap26 have been shown to act in a connexin-specific manner [Chaytor *et al*, 1999; Berman *et al*, 2002] but our findings concur with others who have reported differential activity between these two CMPs. In 2003, Chaytor and Griffith reported that ^{37,43}Gap27 and ^{40}Gap27 partially inhibited the transmission of hyperpolarisation from endothelium to smooth muscle in the rabbit iliac artery. However, the addition of ^{43}Gap26 was required to abolish this phenomenon completely, implying that ^{37,43}Gap27 causes incomplete inhibition of Cx43 [Chaytor *et al*, 2003]. Because each of these peptides shares homology with different extracellular loops, these data

suggest that targeting ^{37,43}Gap27 and ^{43}Gap26 vary in their propensity to inhibit Cx43 by acting at different sites. It is unclear whether Gap26 plays a greater role than Gap27 in Cx43 gating and/or docking.

Immunohistochemistry revealed Cx37 and Cx43 expression in both the endothelium and smooth muscle. Cx40 was also seen in these cells but expression was predominantly seen in the endothelium. Whilst myoendothelial gap junctions are proposed to allow the transfer of hyperpolarisation generated in the endothelium to the underlying smooth muscle, homocellular gap junctions between adjacent smooth muscle or endothelial cells are also an ideal conduit for the longitudinal conduction of hyperpolarisation and coordinated regulation of vascular tone. Indeed, mice genetically engineered to lack vascular Cx40 display not only hypertension but also irregular vasomotion [de Wit *et al*, 2003]. Our own study has not differentiated whether the inhibition of EDHF-mediated vasodilatation is due to the inhibition of Cx43 in myoendothelial junctions, in homocellular junctions, or in both. Irrespective, we have demonstrated a critical role for this connexin in endothelium-dependent vasorelaxation. Furthermore, we have shown that endothelium-independent vasorelaxation is unaffected by gap junction and, specifically, Cx43 inhibition.

3.6 CONCLUSION

In summary, we have shown that EDHF accounts for approximately 50% of bradykinin-induced vasorelaxation in resistance arteries from pregnant women. Using CMPs, we have provided functional evidence that EDHF mediates its vasorelaxatory actions via gap junctions. We have demonstrated the expression of

three major connexin isoforms and highlighted a predominant functional role for Cx43 in the mediation of EDHF responses in this vascular bed. The extension of these studies to men, non-pregnant women and in pathophysiological states warrants further investigation.

CHAPTER 4

THE VASCULAR EFFECTS OF ROTIGAPTIDE

IN VIVO IN MAN

Lang NN, Myles RP, Burton FL, Hall DP, Chin YZ, Boon NA, Newby DE.
The Vascular Effects of Rotigaptide *In Vivo* in Man.
Biochem Pharmacol (in Press).

4.1 SUMMARY

Endothelium-derived hyperpolarising factor causes vasorelaxation and may contribute to the release of the endogenous fibrinolytic factor, t-PA. Rotigaptide enhances communication via the connexin 43 gap junction subunit and may potentiate the vascular actions of EDHF. The aims of this study were to determine whether rotigaptide influences basal and stimulated endothelium-dependent vasodilatation and t-PA release *in vivo* in man. Using venous occlusion plethysmography, forearm blood flow was measured in 27 healthy volunteers during intra-brachial infusions of rotigaptide (0.25–25 nmol/min) alone, or co-administered with endothelium-dependent (acetylcholine [5-20 μg/min] and bradykinin [30-300 pmol/min]) and independent (sodium nitroprusside [2-8 μg/min]) vasodilators in the presence or absence of the 'nitric oxide clamp' and aspirin. Basal blood flow was unaffected by rotigaptide (P=NS). Acetylcholine, bradykinin and sodium nitroprusside all caused dose-dependent vasodilatation in the presence and absence of aspirin and the 'nitric oxide clamp' ($P \leq 0.005$ for all). These responses were unaffected by rotigaptide (P=NS). Bradykinin caused t-PA antigen and activity release (P=0.04, $P<0.0001$, respectively) that was unaffected by rotigaptide. These results demonstrate that augmentation of connexin 43 communication has no effect on basal vascular tone and does not enhance endothelium-dependent or independent vasodilatation, or t-PA release in the forearm arterial circulation of healthy men. It remains to be established whether augmentation of connexin 43 communication improves endothelial function in patients with vascular disease.

4.2 INTRODUCTION

The endothelium plays a major role in the regulation of vascular tone and is responsible for the release of the endogenous fibrinolytic factor, t-PA. The elucidation of their roles in vascular physiology and pathophysiology has been fundamental to recent advances in the treatment and prevention of many cardiovascular diseases.

After blockade of both NO and PGI_2 generation, a substantial degree of endothelium-dependent vasodilatation remains and is attributed to EDHF [Vanhoutte, 1987]. Consistently, its contribution is most prominent in the small resistance arteries [Urakami-Harasawa *et al*, 1997; Berman *et al*, 2002] that regulate systemic blood pressure and local tissue perfusion. Furthermore, the release of t-PA from the endothelium is independent of both NO and PGI_2 production [Labinjoh *et al*, 2000; Hrafnkelsdottir *et al*, 2001] and it has been suggested that EDHF may mediate its release [Brown *et al*, 2000; Hrafnkelsdottir *et al*, 2001]. Thus, alterations in EDHF activity may contribute to endothelial dysfunction and its manipulation presents an exciting opportunity to restore vascular health and reduce the burden of cardiovascular disease [Feletou and Vanhoutte, 2004].

The transmission of hyperpolarisation from the endothelium to the underlying smooth muscle occurs via undefined mediators and pathways. Various mediators have been proposed but none has emerged as a universal EDHF in all species and vascular beds [Griffith, 2004; Feletou and Vanhoutte, 2006a]. However, there is

considerable evidence that gap junctions are required [Chaytor *et al*, 1998; Rummery *et al*, 2002; de Wit *et al*, 2003; Griffith, 2004]. These aqueous pores are found at points of cell-cell contact and allow the intercellular transfer of small molecules (<1 kDa). Myoendothelial gap junctions are, therefore, ideally suited to the radial transfer of electrotonic charge or second messenger between the endothelium and underlying smooth muscle. Each gap junction hemichannel is composed of six connexin subunits including Cx37, 40 and 43 in the mammalian vasculature. Indeed, using specific connexin antagonist peptides, we have previously demonstrated the critical importance of Cx43 in EDHF-mediated vasodilatation of human resistance arteries *in vitro* [Lang *et al*, 2007].

It has not previously been possible to make a direct assessment of the role of gap junctions in the mediation of EDHF responses *in vivo* in man. However, rotigaptide is a synthetic hexapeptide (Ac-D-Tyr-D-Pro-D-Hyp-Gly-D-Ala-Gly-NH_2) that acts upon Cx43 to potentiate communication via gap junctions [Xing *et al*, 2003; Clarke *et al*, 2006; Axelsen *et al*, 2007; Kjølbye *et al*, 2008]. In addition to its clinical development as an antiarrhythmic agent [Kjolbye *et al*, 2003; Udata *et al*, 2006], it has been promoted as an important new tool to aid in the dissection of the physiologic role of gap junctions [Axelsen *et al*, 2007]. Here, we have conducted the first clinical assessment of the role of gap junctions, and specifically Cx43, in the peripheral vascular EDHF-mediated responses. We tested the hypothesis that, by increasing communication via gap junctions, rotigaptide would enhance EDHF-mediated vasodilatation and t-PA release in the forearm arterial circulation of healthy man.

4.3 METHODS

4.3.1 PRELIMINARY VALIDATION STUDY

The biological activity of rotigaptide was assessed through *in vitro* measurements of its effect on transmural conduction velocity in rabbit ventricular myocardium. Hearts from four male New Zealand white rabbits were used for these experiments, which conform to the standards set out in the Animals (Scientific Procedures) Act 1986.

Rabbits (n=4) were killed with a single intravenous injection of 100 mg/kg pentobarbital sodium. Hearts were excised, placed in chilled Tyrode's solution (containing [mmol/L]: Na 134.5, Mg 1.0, K 5.0, Ca 1.9, Cl 101.8, SO_4 1.0, H_2PO_4 0.7, HCO_3 20, acetate 20 and glucose 10) and perfused via the left coronary artery with oxygenated (95% O_2-5% CO_2) Tyrode's solution maintained at pH 7.4 and 37°C. Perfused left ventricular free wall wedge preparations were dissected out and mounted in a custom built chamber which allowed access to the transmural surface for imaging. The preparation was stimulated using a bipolar electrode placed on the epicardial surface at 1.5 x diastolic threshold using a 2 ms pulse at a basic cycle length of 350 ms. Perfusion pressure and electrocardiogram were monitored throughout. An optical mapping system was used to record optical action potentials as previously described [Walker *et al*, 2007]. Motion artefact was minimised using 15 mmol/L 2,3 butanendione monoxime. Measurements were taken at 15-minute intervals. After two control recordings the perfusate was changed to Tyrode's solution containing butanendione monoxime and 1 μmol/L rotigaptide for a further 15 minutes and measurements were repeated.

4.3.2 CLINICAL STUDY

This clinical study was performed with the approval of the local Research Ethics Committee in accordance with the Declaration of Helsinki and with the written informed consent of each subject.

Subjects

Healthy non-smokers (mean age 22 years; range 19-25 years) were recruited into the study. Participants were excluded if they had 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. On the day of study, participants had fasted and abstained from caffeine and tobacco for at least 4 hours and from alcohol for 24 hours.

Drugs

Pharmaceutical grade bradykinin (Clinalfa AG, Läufelfingen, Switzerland), acetylcholine (Novartis Ltd, Middlesex, UK), L-N(G)-monomethyl arginine citrate (Clinalfa), sodium nitroprusside (Hospira Inc., CA, USA) and rotigaptide (American Peptide Inc., CA., USA) were dissolved in physiological saline. Aspirin was obtained from Dagra Pharma, Diemen, Netherlands.

Forearm Venous Occlusion Plethysmography

All subjects underwent cannulation of the brachial artery with a 27-gauge standard wire steel needle under controlled conditions. All studies were performed with patients lying supine in a quiet, temperature controlled (22-25 °C) room. 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.

Intra-Arterial Drug Administration

PROTOCOL 1: Effect of rotigaptide on basal forearm blood flow

Five participants attended once each. They received intra-arterial rotigaptide at 0.25, 0.75, 2.5, 7.5 and 25 nmol/min for 6 minutes at each dose. Assuming basal FBF of 25 mL/min, this would give tissue rotigaptide concentrations of 0.01-1.0 μmol/L. Forearm blood flow was measured during the final 3 minutes of infusion of each dose.

PROTOCOL 2: Effect of rotigaptide on agonist-induced vasodilatation and tissue plasminogen activator release

Twelve volunteers attended on each of three occasions. After a 20-minute intra-arterial infusion of 0.9% saline, participants received either intra-arterial placebo (0.9% saline), 2.5 nmol/min rotigaptide or 25 nmol/min rotigaptide using a

randomised double-blind crossover design. Rotigaptide or placebo was administered alone for 20 minutes before being co-infused with ascending doses of bradykinin (an endothelium-dependent vasodilator that causes the release of t-PA; 30-300 pmol/min), acetylcholine (an endothelium-dependent vasodilator that does not cause the release of t-PA; 5-20 μg/min) and sodium nitroprusside (an endothelium-independent vasodilator that does not cause the release of t-PA; 2-8 μg/min). Co-infused drugs were separated by a 20-minute infusion of 0.9% saline. The order of agonist infusion was randomised between participants but maintained constant for each of the three visits.

PROTOCOL 3: Effect of rotigaptide on EDHF-mediated vasodilatation

Ten volunteers were recruited to attend on two occasions. Endothelium-derived hyperpolarising factor activity was isolated by inhibiting the production of both PGI_2 and NO on each of the two visits. Cyclooxygenase activity was inhibited with a single 600 mg dose of oral aspirin 1 hour prior to each study. Nitric oxide production was inhibited with L-NMMA in the 'nitric oxide clamp'. After a 20-minute intra-arterial infusion of 0.9% saline, L-NMMA (8 μmol/min) was infused via the brachial artery. To compensate for L-NMMA induced basal vasoconstriction, FBF was restored to baseline using a titrated dose of exogenous NO in the form of intra-brachial sodium nitroprusside (90-900 ng/min). The titrated dose of sodium nitroprusside was co-infused with L-NMMA throughout the study. This arrangement allows a constant 'clamped' delivery of exogenous NO whilst endogenous NO synthase activity is inhibited [Honing *et al*, 2000].

Either rotigaptide (25 nmol/min) or saline placebo was co-infused with the 'nitric oxide clamp' in a double-blind randomised crossover design. Subsequently, ascending doses of bradykinin (30-300 pmol/min), acetylcholine (5-20 μg/min) and sodium nitroprusside (2-8 μg/min) were co-infused and separated by a 20-minute infusion of 0.9% saline. The order of agonist co-infusion was randomised between participants but maintained constant between visits.

Blood Sampling

Seventeen-gauge venous cannulae were inserted into left and right antecubital fossae during Protocol 2. Blood samples were drawn simultaneously from each arm before bradykinin infusion and after the maximum dose of bradykinin (300 pmol/min). Blood was collected into acidified buffered citrate (Stabilyte, Biopool International, UK; for t-PA assays) and into citrate (BD Vacutainer, BD UK Ltd, UK; for measurement of t-PA's major endogenous inhibitor, PAI-1). 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. Haematocrit was measured at baseline and at the end of the study.

4.3.5 DATA ANALYSIS AND STATISTICS

Optical Mapping Validation Study

Data analysis was performed using custom software. Activation time was determined at the midpoint between baseline and the peak of the action potential upstroke. Transmural conduction velocity was calculated for each time point using activation time from the earliest activation on the epicardial edge of the transmural surface to the earliest activation on the endocardial side.

Clinical Study

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

4.4 RESULTS

4.4.1 EFFECT OF ROTIGAPTIDE ON MYOCARDIAL CONDUCTION VELOCITY *IN VITRO*

In all experiments, transmural conduction velocity remained constant during control perfusion (20.8±1.7 *vs.* 21.4±1.6 cm/s, P=NS). Following perfusion with rotigaptide, an increase in transmural conduction velocity was observed (29.4±1.7 cm/s, P<0.001, ANOVA; Figure 4.1).

4.4.2 EFFECT OF ROTIGAPTIDE ON BASAL FOREARM BOOD FLOW

At doses of 0.25-25 nmol/min, rotigaptide had no effect upon basal FBF (P=NS, ANOVA; Figure 4.2).

4.4.3 EFFECT OF ROTIGAPTIDE ON AGONIST-INDUCED VASODILATATION AND TISSUE PLASMINOGEN ACTIVATOR RELEASE

Acetylcholine, bradykinin and sodium nitroprusside each caused dose-dependent arterial vasodilatatation (P<0.0001 for all, ANOVA) that was unaffected by either 2.5 or 25 nmol/min rotigaptide (P=NS for both, in the presence vs. the absence of rotigaptide, ANOVA; Figure 4.3).

At baseline, net t-PA antigen release was 0.09±0.05 ng/100mL/min and net release of t-PA activity was 0.54±1.4 IU/100 mL/min. This was unchanged by the presence of rotigaptide (P=NS for all, ANOVA). Bradykinin (300 pmol/min) caused the release of t-PA antigen (7.62±5.56 ng/100 mL/min; P=0.04, ANOVA) and activity

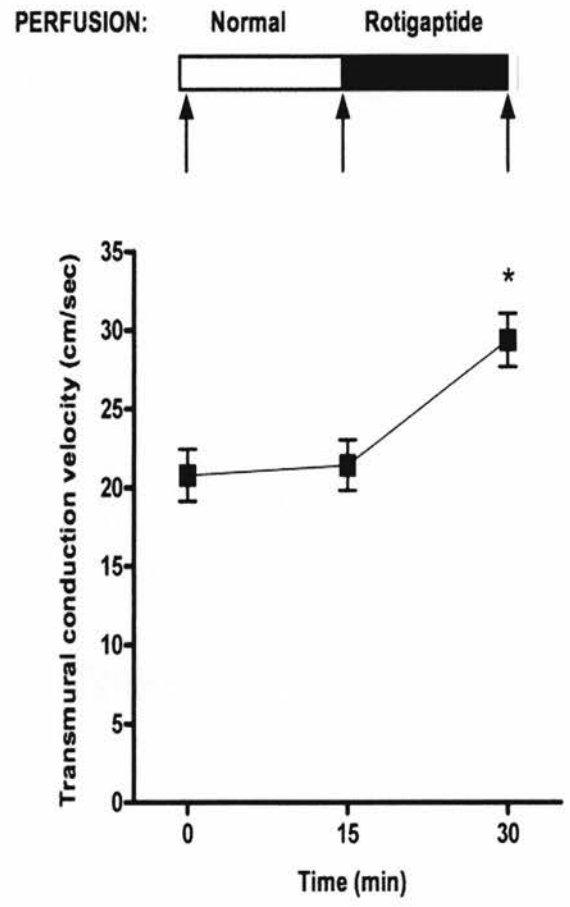


Figure 4.1 *Effect of 1 μmol/L rotigaptide on transmural conduction velocity in perfused rabbit ventricular myocardium.*
**P<0.001, 30 minutes vs. 0 or 15 minutes, t-test.*

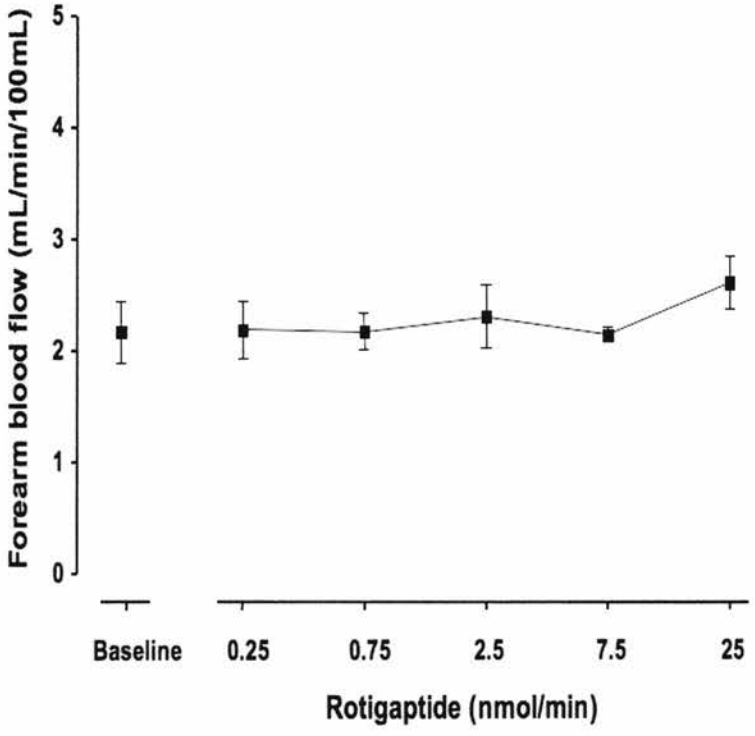


Figure 4.2 *Forearm blood flow during intra-brachial infusion of rotigaptide (0.25–25 nmol/min).*

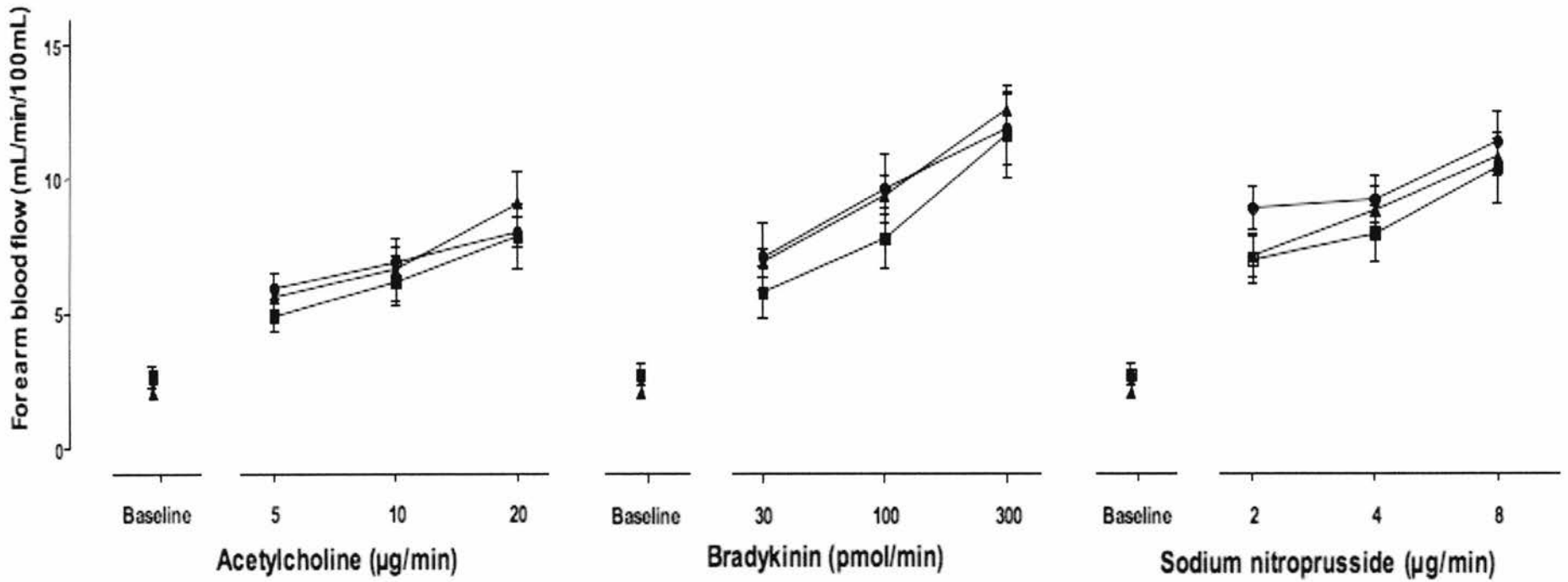


Figure 4.3 *Forearm blood flow during intra-brachial infusion of acetylcholine (left hand panel), bradykinin (middle panel) and sodium nitroprusside (right hand panel) in the presence of placebo (squares), 2.5 nmol/min rotigaptide (triangles) or 25 nmol/min rotigaptide (circles). P=NS for rotigaptide vs. placebo; Analysis of variance (ANOVA).*

(6.15±2.20 IU/100 mL/min; P<0.0001, ANOVA) but this was unaffected by either 2.5 or 25 nmol/min rotigaptide (P=NS for all, ANOVA).

Net release of PAI-1 antigen and activity was unaffected by bradykinin (300 pmol/min) in the presence or absence of rotigaptide (P=NS for all, ANOVA; data not shown).

4.4.4 Effect of rotigaptide on EDHF-mediated vasodilatation

Intra-arterial L-NMMA (8 μmol/min) reduced basal FBF by approximately 38% (from 2.40±0.20 reduced to 1.50±0.20 mL/100 mL tissue/min; P<0.0001) and was unaffected by rotigaptide (P=NS, 2-way ANOVA). Forearm blood flow was restored to baseline levels by the titration of sodium nitroprusside (P=NS, ANOVA).

After the inhibition of endothelial NO and PGI_2 synthesis, dose-dependent vasodilatation was evoked by acetylcholine, bradykinin and sodium nitroprusside (P≤0.005 for all, ANOVA). This response was not altered by the presence of rotigaptide (P=NS, ANOVA; Figure 4.4).

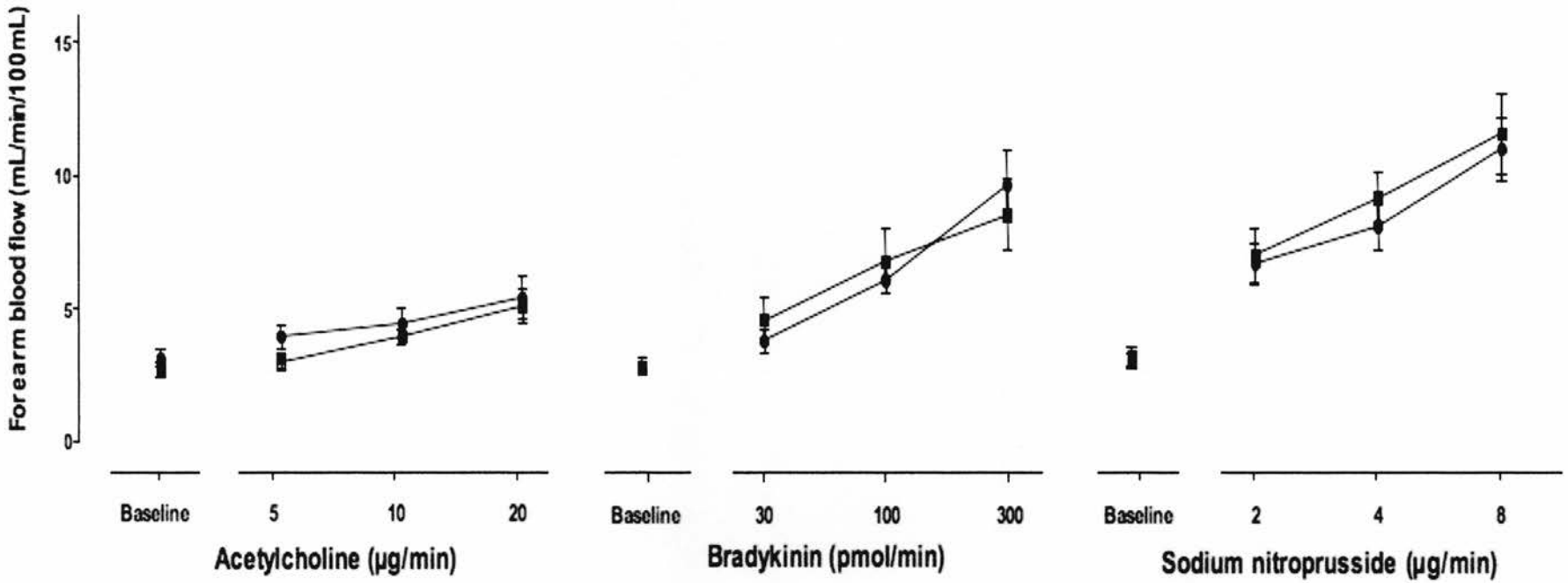


Figure 4.4 *In the presence of the 'nitric oxide clamp' and oral aspirin, forearm blood flow during intra-brachial infusion of acetylcholine (left hand panel), bradykinin (middle panel) and sodium nitroprusside (right hand panel) in the presence of placebo (squares) or 25 nmol/min rotigaptide (circles). P=NS for rotigaptide vs. placebo; Analysis of variance (ANOVA).*

4.5 DISCUSSION

For the first time, we have assessed the role of connexin 43 and gap junctions in the control of vascular tone and t-PA release *in vivo* in man. We have shown, in healthy volunteers, that potentiation of Cx43-mediated intercellular communication with rotigaptide does not affect basal forearm arterial blood flow or agonist-induced vasodilatation and t-PA release in the presence or absence of concomitant inhibition of PGI_2 and NO production.

To date, there has been no direct assessment of the role of gap junctions, nor individual connexin subtypes, in the regulation of vascular function *in vivo* in man. The use of connexin antagonists has been limited by concerns about the potential for toxicity and older putative gap junction blockers exert a range of non-endothelial effects and alter ion channel permeability [Chaytor *et al*, 2000; Matchkov *et al*, 2004]. However, rotigaptide offers a novel means for the assessment of gap junction-dependent phenomena *in vivo*. Not only has it been safely administered to healthy humans [Kjolbye *et al*, 2003; Udata *et al*, 2006] but its actions are specific to Cx43. Indeed, it augments communication via gap junctions in the absence of changes in membrane conduction or basal current [Muller *et al*, 1997], and it exhibits no binding to a large array of receptors including numerous ion channels [Haugan *et al*, 2005]. It enhances intercellular dye transfer between HeLa cells expressing Cx43 but not between those expressing Cx26 or Cx32 [Clarke *et al*, 2006] and promotes electrical coupling between ventricular myocytes via alterations in the phosphorylation status of Cx43 [Axelsen *et al*, 2006; Kjølbye *et al*, 2008]. Indeed, phosphorylation of Cx43 maintains it an open state [Ek-Vitorin *et al*, 2006]

and Kjølbye and co-workers have recently demonstrated that the antiarrhythmic effects of rotigaptide are associated with the inhibition of Cx43 dephosphorylation [Kjølbye *et al*, 2008].

We have previously demonstrated that EDHF-mediated vasodilatation of subcutaneous resistance arteries from pregnant women depends upon the presence of functional Cx43 whilst Cx37 and Cx40 are not required [Lang *et al*, 2007]. Indeed, in comparison to Cx37 and Cx40, the vascular expression of Cx43 appears to be particularly labile. It is up-regulated by oestrogen [Liu *et al*, 2002], at the leading edge of atherosclerotic plaques [Kwak *et al*, 2002], after vascular injury [Yeh *et al*, 1997] and at sites of increased shear stress [Gabriels and Paul, 1998; Depaola *et al*, 1999]. Furthermore, type I diabetes mellitus is associated with an impairment of EDHF-mediated vascular responses [Feletou and Vanhoutte, 2004; Fitzgerald *et al*, 2005], and elevated glucose concentrations cause the isolated down-regulation of Cx43 expression [Sato *et al*, 2002] and permeability [Kuroki *et al*, 1998] *in vitro*. The examination of Cx43-mediated responses is, therefore, of particular relevance not only to our understanding of vascular physiology, but might also represent a major therapeutic target to attenuate endothelial dysfunction and improve vascular health.

Despite theoretical indications, we have failed to demonstrate a major vascular effect of rotigaptide even during inhibition of both NO and PGI_2 production. Was the rotigaptide inactive or used at an inadequate dose? Using an electrophysiological optical mapping system, the biological activity of rotigaptide at 1 μmol/L was

confirmed. The maximum dose of rotigaptide assessed in the clinical study was 25 nmol/min which, based upon FBF of 25 mL/min, equates to an estimated tissue concentration of approximately 1 μmol/L. This concentration is in excess of the effective antiarrhythmic concentration employed in previous animal models [Xing *et al*, 2003; Guerra *et al*, 2006; Hennan *et al*, 2006; Shiroshita-Takeshita *et al*, 2007] and is similar to the maximum tissue concentration of rotigaptide achieved in clinical trials [Udata *et al*, 2006]. Furthermore, it is in excess of the concentration recently shown to be required for the prevention of Cx43 dephosphorylation [Kjølbye *et al*, 2008]. We therefore do not believe that the preparation was inactive or used at the wrong dose.

Connexin 43 is expressed abundantly in a wide range of resistance arteries obtained from a variety of species [Griffith, 2004] including humans [Lang *et al*, 2007]. Whilst our previous assessment of the role of connexin subtypes in human EDHF-mediated responses demonstrated a critical role for Cx43 and not Cx37 or 40 [Lang *et al*, 2007], it is possible that, in the non-pregnant state, the vasomotor and EDHF-mechanism requires a contribution from all of these subtypes. Rotigaptide specifically augments Cx43-mediated signalling but the augmentation of vascular gap junction mediated communication may therefore require the combined potentiation of all three of the major vascular connexin subtypes.

The present study made an examination of vascular responses in healthy young men. It is conceivable that vasomotor and endogenous fibrinolytic responses cannot be augmented because the endothelium is already maximally active with a high baseline

open-state probability for Cx43. Indeed, the antiarrhythmic activity of rotigaptide is particularly potent in the context of acidosis and metabolic stress during which the open-state probability of Cx43 is relatively low [Haugan *et al*, 2005]. Furthermore, we were careful to dissect out EDHF-mediated activity by the inhibition of PGI_2 and NO with oral aspirin and the 'nitric oxide clamp' respectively. EDHF has activity that is reciprocal to NO [Bauersachs *et al*, 1996] and becomes up-regulated to compensate for impaired NO bioavailability in a variety of disease states [Feletou and Vanhoutte, 2004]. Therefore, the argument that EDHF is maximally active in the studied population becomes more pertinent in the presence of NO and PGI_2 inhibition.

4.6 CONCLUSION

In conclusion, we have demonstrated that intra-arterial rotigaptide does not augment vasomotion or endogenous fibrinolysis in healthy subjects. Whether enhancement of connexin 43-mediated intercellular communication influences vascular function in conditions associated with specific impairment of EDHF-mediated activity, such as type I diabetes mellitus or pre-eclampsia, remains to be evaluated. Not only would this assessment provide important mechanistic insights to the pathophysiology of endothelial dysfunction but could also highlight an important novel therapeutic target.

CHAPTER 5

ROLE OF THE ENDOTHELIUM IN THE VASCULAR EFFECTS OF THE THROMBIN RECEPTOR (PROTEASE-ACTIVATED RECEPTOR TYPE 1) IN HUMANS

Guðmundsdóttir IJ, **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.
J Am Coll Cardiol 2008;51:1749-1756.

5.1 SUMMARY

Thrombin is central to the pathophysiology of atherothrombosis and its cellular actions are mediated via PAR-1. Activation of PAR-1 causes arterial vasodilatation, venoconstriction, platelet activation and t-PA release in man. The objective 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 in six healthy volunteers before and after endothelial denudation. Forearm arterial blood flow and plasma fibrinolytic factors were measured in 24 healthy volunteers during venous occlusion plethysmography. The effects of inhibition of PGI_2, NO and endothelium-derived hyperpolarising factor 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. These results show 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 autoactivates 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].

PAR-1 receptors 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, mitogen-activated protein kinases and potassium channels [Coughlin, 2000; Ossovskaya and Bunnett, 2004]. 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 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 receptor agonist, 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, the *in vivo* effects of PAR-1 activation in platelets, endothelium and vascular smooth muscle in man have recently been described by Guðmundsdóttir *et al*. For the first time, it was shown 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 local 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.

5.3.2 VASCULAR ASSESSMENTS

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.

5.3.3 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 linear variable differential transformer, 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-NH_2 (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*, 2000]. 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*, 2000]. 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 reattended 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, FBF 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 crossover study employing a two-by-two factorial design: with and without aspirin

(600 mg orally; to inhibit PGI_2 synthesis) and the 'nitric oxide clamp'. Assuming total FBF 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, FBF 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.

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 microg/min) were co-infused with either saline placebo or TEA (1 mg/min) on either of two visits using a randomised double-blind crossover 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 and Kadowitz, 1997; Honing *et al*, 2000; Inokuchi *et al*, 2003].

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 crossover design. In this series, EDHF activity was isolated by inhibiting NO and PGI_2 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 (BD Vacutainer, BD UK Ltd, UK; for PAI-1, beta 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), beta 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.

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. After preconstriction 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 *vs.* 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).

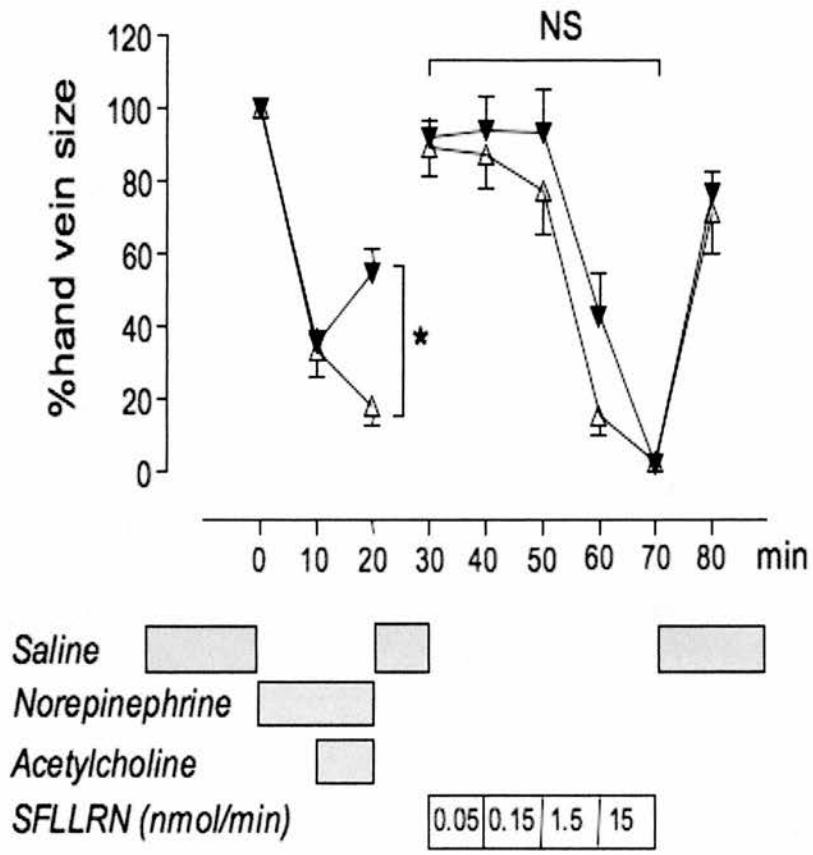


Figure 5.1 *Dorsal hand vein responses to acetylcholine and SFLLRN (after preconstriction with norepinephrine) Before (closed triangles) and after (open triangles) endothelial denudation.*
**P<0.05 and NS=non-significant (P=0.09); presence vs. absence of the endothelium; Analysis of variance (ANOVA).*

5.4.2 ENDOTHELIUM-DERIVED VASODILATORS AND PAR-1 INDUCED VASODILATATION

SFLLRN caused an increase in FBF that was unaffected by PGI_2 inhibition with oral aspirin (600 mg; Figure 5.2A). The NO synthase inhibitor, L-NMMA, caused ~50% decrease in basal FBF 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 FBF back to baseline levels (2.77±0.24 mL/100mL tissue/min; P=0.46 clamp dose SNP *vs.* 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 PGI_2, NO synthase and potassium channel inhibition appeared to abolish SFLLRN-induced vasodilatation (Figure 5.2D).

Both with and without concurrent NO synthase and PGI_2 synthase inhibition, TEA attenuated, but did not abolish arterial vasodilatation to the control endothelium-dependent vasodilator, bradykinin (Figure 5.3A and 5.3B). Tetraethylammonium ion did not affect endothelium-independent forearm arterial vasodilatation to sodium nitroprusside (Figure 3C).

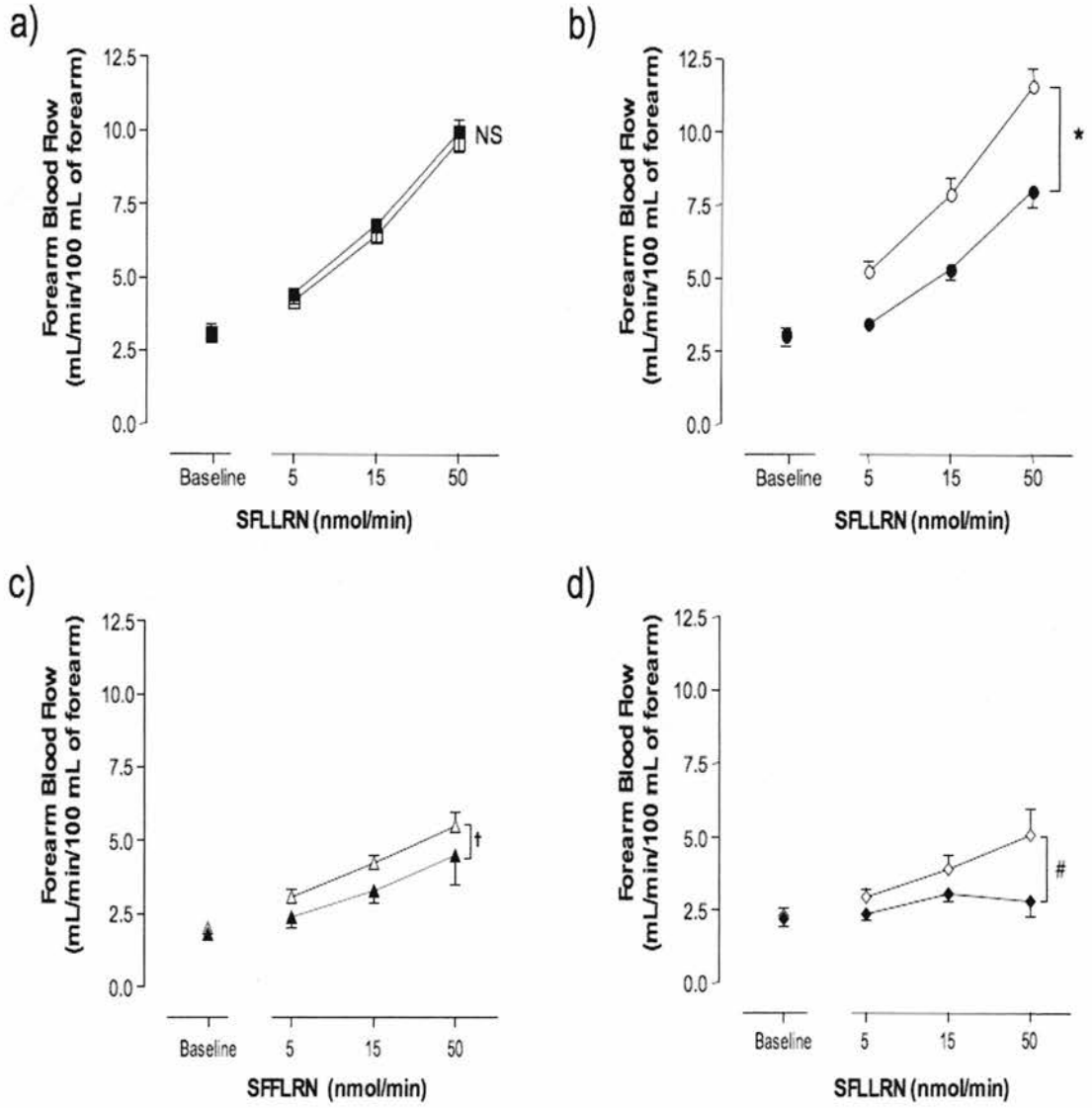


Figure 5.2 *SFLLRN-induced forearm arterial vasodilatation. In the presence (closed symbols) and absence (open symbols) of A) aspirin (squares); NS=non-significant (P=0.53) in the presence vs. the absence of aspirin; Analysis of variance (ANOVA), B) the 'nitric oxide (NO) clamp' (circles); *P<0.0001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

#P<0.01 in the presence vs. the absence of aspirin, the 'NO clamp' and TEA (ANOVA).

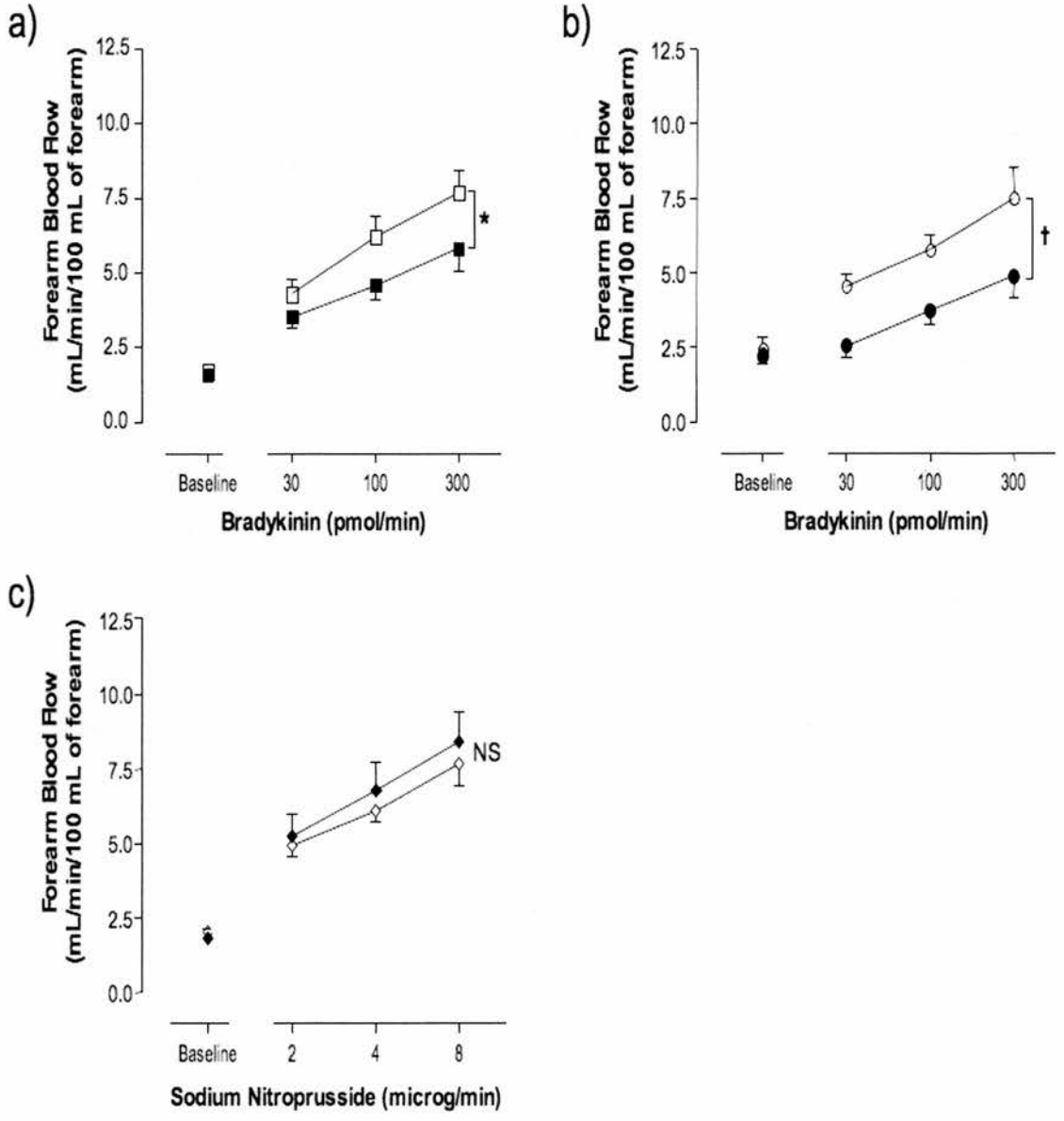


Figure 5.3 *Bradykinin- and sodium nitroprusside-induced forearm arterial vasodilatation. Forearm arterial vasodilatation induced by (A) bradykinin (squares), (B) bradykinin in the presence of the 'nitric oxide clamp' and aspirin (circles) and (C) sodium nitroprusside (diamonds), in the presence (closed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

5.4.3 ENDOTHELIUM-DERIVED VASODILATORS AND PAR-1 INDUCED RELEASE OF FIBRINOLYTIC, COAGULANT AND PLATELET 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). TEA did not alter bradykinin induced PAI-1 or t-PA release (P=NS for both, ANOVA). Unpaired analysis between the two subject groups (Protocol 4 *vs.* Protocol 5) suggests that the 'nitric oxide 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). SFLLRN increased beta-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).

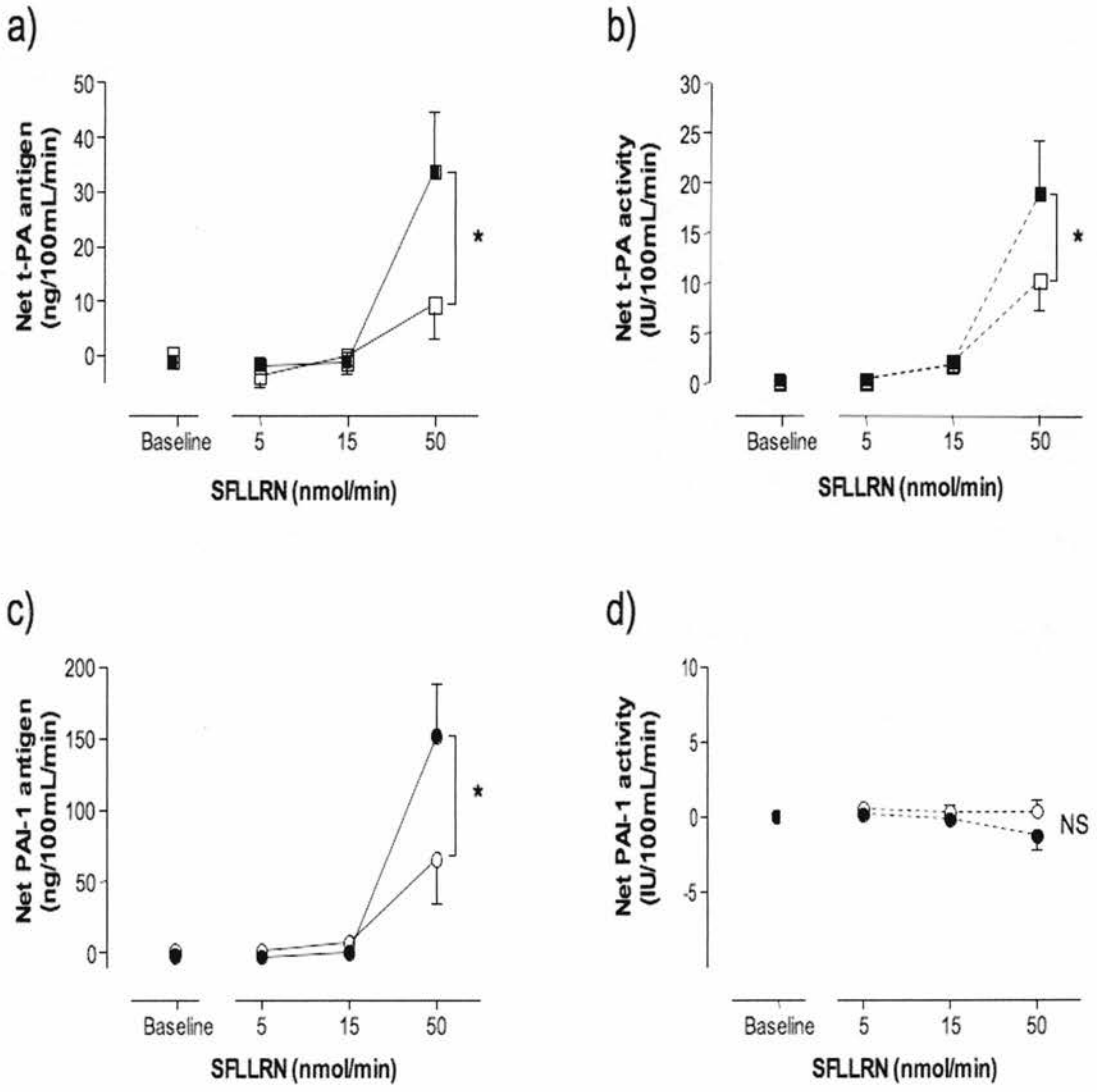


Figure 5.4 *SFLLRN-induced tissue type plasminogen activator and plasminogen activator inhibitor release. Net tissue plasminogen activator (t-PA; squares) and plasminogen activator inhibitor type 1 (PAI-1; circles) antigen (solid lines) and activity (dashed lines) in response to intra-brachial SFLLRN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

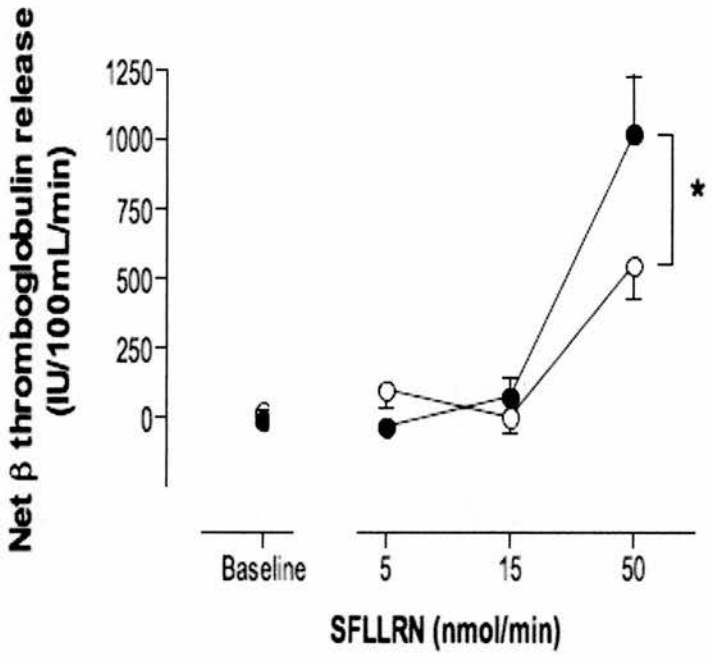


Figure 5. 5 *SFLLRN-induced beta-thromboglobulin release. In the presence (closed circles) and absence (open circles) of the 'nitric oxide (NO) clamp'.*
**P<0.01 net beta-thromboglobulin release induced by SFLLRN (50 nmol/min) in the presence vs. the absence of the 'NO clamp', analysis of variance (ANOVA).*

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 here described 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

The unexpected finding of PAR-1 induced venoconstriction in man has previously been described by [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 NO. 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: PGI_2, NO and EDHF. Although PGI_2 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 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*

[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. However, in contrast to TEA's 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 [Muldowney *et al*, 2007] 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 whilst antagonists of specific epoxyeicosatrienoic acids 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 the concomitant release of beta-thromboglobulin which 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 anti-platelet 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 *vs.* 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, Guðmundsdóttir *et al* 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*, 2006]. 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; Ossovskaya and Bunnett, 2004].

The role of NO in bradykinin-induced t-PA release remains controversial [Oliver *et al,* 2005] and the present study has not definitively addressed this issue. Whilst NO donors do not induce t-PA release [Newby *et al*, 1997b; Stein *et al*, 1998], inhibition of NO synthesis has been reported either to have no effect [Smith *et al,* 2003], or to increase [Brown *et al*, 2001], bradykinin-induced t-PA release. Our own unpaired analysis of data from different subject populations is in keeping with the findings of Nancy Brown's group [Brown *et al,* 2001] and suggests that bradykinin-induced t-PA release is unaffected by either NO or PGI_2 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.

5.6 CONCLUSION

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

MARKED IMPAIRMENT OF PAR-1 MEDIATED VASODILATATION AND FIBRINOLYSIS IN CIGARETTE SMOKERS

Lang NN, Guðmundsdóttir IJ, Boon NA, Ludlam CA, Fox KA, Newby DE.
Marked Impairment of PAR-1 Mediated Vasodilatation
and Fibrinolysis in Cigarette Smokers.
J Am Coll Cardiol 2008;**52**:33-39.

6.1 SUMMARY

The objective of this study was 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 12 cigarette smokers and 12 age- and sex-matched non-smokers during intra-brachial 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 intervals throughout. All agonists caused dose-dependent increases in FBF ($P<0.0001$ 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). These results demonstrate 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. These findings suggest a major contribution of impaired endothelial PAR-1 action to the increased atherothrombotic risk of 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 recent 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*, 2005]. 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].

Protease activated receptor type 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, it has been have shown that acting via PAR-1 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].

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 we hypothesised 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 non-smoking control subjects.

6.3 MATERIALS AND METHODS

6.3.1 SUBJECTS

Twelve healthy cigarette smokers (5-20 cigarettes/day) and 12 age- and sex-matched non-smokers between 20 and 46 years old 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, 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 non-steroidal 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°C to 24°C.

6.3.2 ARTERIAL DRUG ADMINISTRATION

All subjects underwent brachial artery cannulation with a 27-gauge standard wire 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 glycoprotein IIb/IIIa antagonist, tirofiban (1.25 μg/min; Merck, Sharp and Dohme, Hoddesdon, UK), was infused and continued throughout the study to inhibit potential PAR-1 induced platelet aggregation. This dose of tirofiban does not affect FBF [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; Clinalfa, Läufelfingen, Switzerland), bradykinin (an endothelium-dependent vasodilator that causes the release of t-PA; 100, 300 and 1000 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, UK). 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 (BD 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.

6.3.3 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 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±1 pack years).

6.4.1 FOREARM BLOOD FLOW

Tirofiban did not affect FBF (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).

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

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

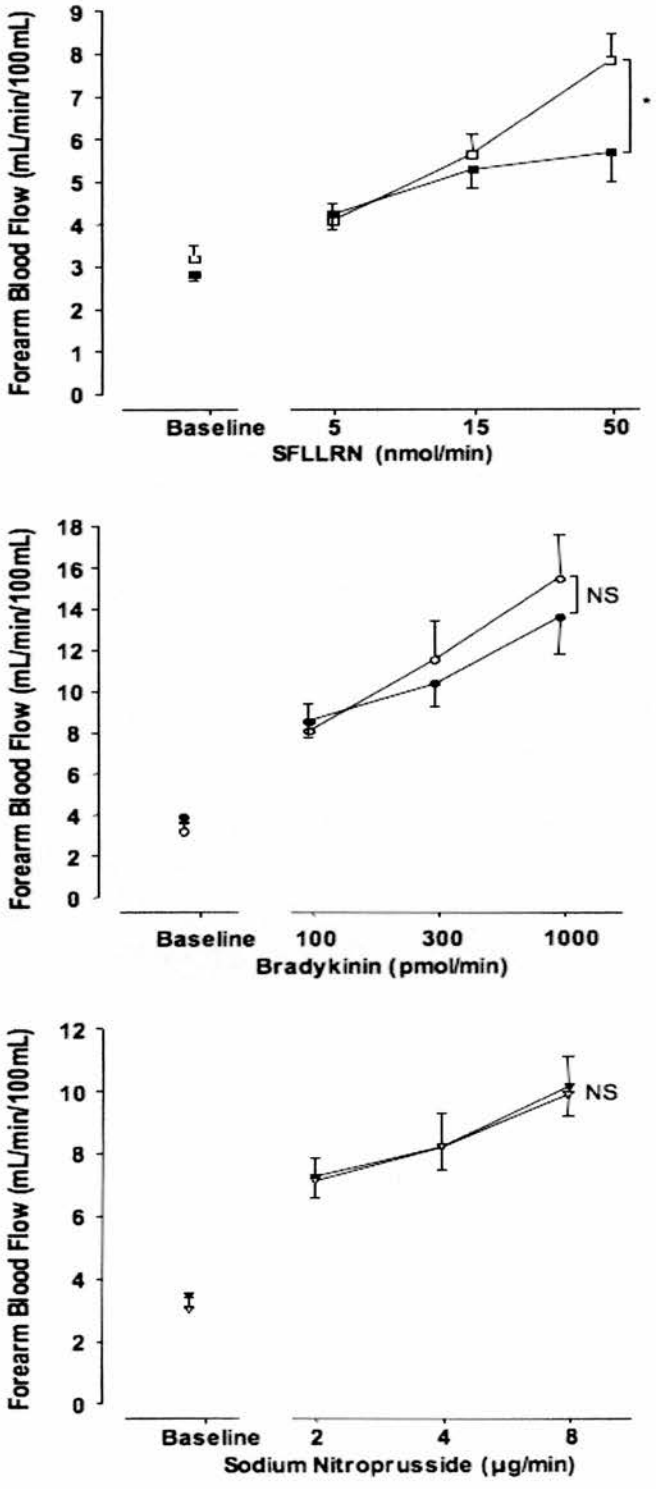


Figure 6.1 *Forearm arterial vasodilatation in smokers and non-smokers. Forearm arterial vasodilatation induced by SFLLRN (squares), bradykinin (circles) sodium nitroprusside (triangles) in smokers (closed symbols) and non-smokers (open symbols).*
**P<0.05, NS=non-significant; Analysis of variance (ANOVA),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

6.4.2 PLASMA FIBRINOLYTIC AND HAEMOSTATIC FACTORS

Baseline plasma t-PA antigen and activity (Table 6.2) and vWF antigen (data not shown) 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 *vs.* 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 6.3).

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 or vWF antigen release (P=NS for all, ANOVA [data not shown]).

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 *vs.* non-smokers, ANOVA; Figure 6.2). Bradykinin caused no change in net

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 vs. non-smokers.
Tissue-type plasminogen activator - t-PA; Bradykinin - BK.

Table 6.3 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 *vs.* non-smokers.
Plasminogen activator inhibitor type 1 - PAI-1; Bradykinin – BK; Non-significant – NS.

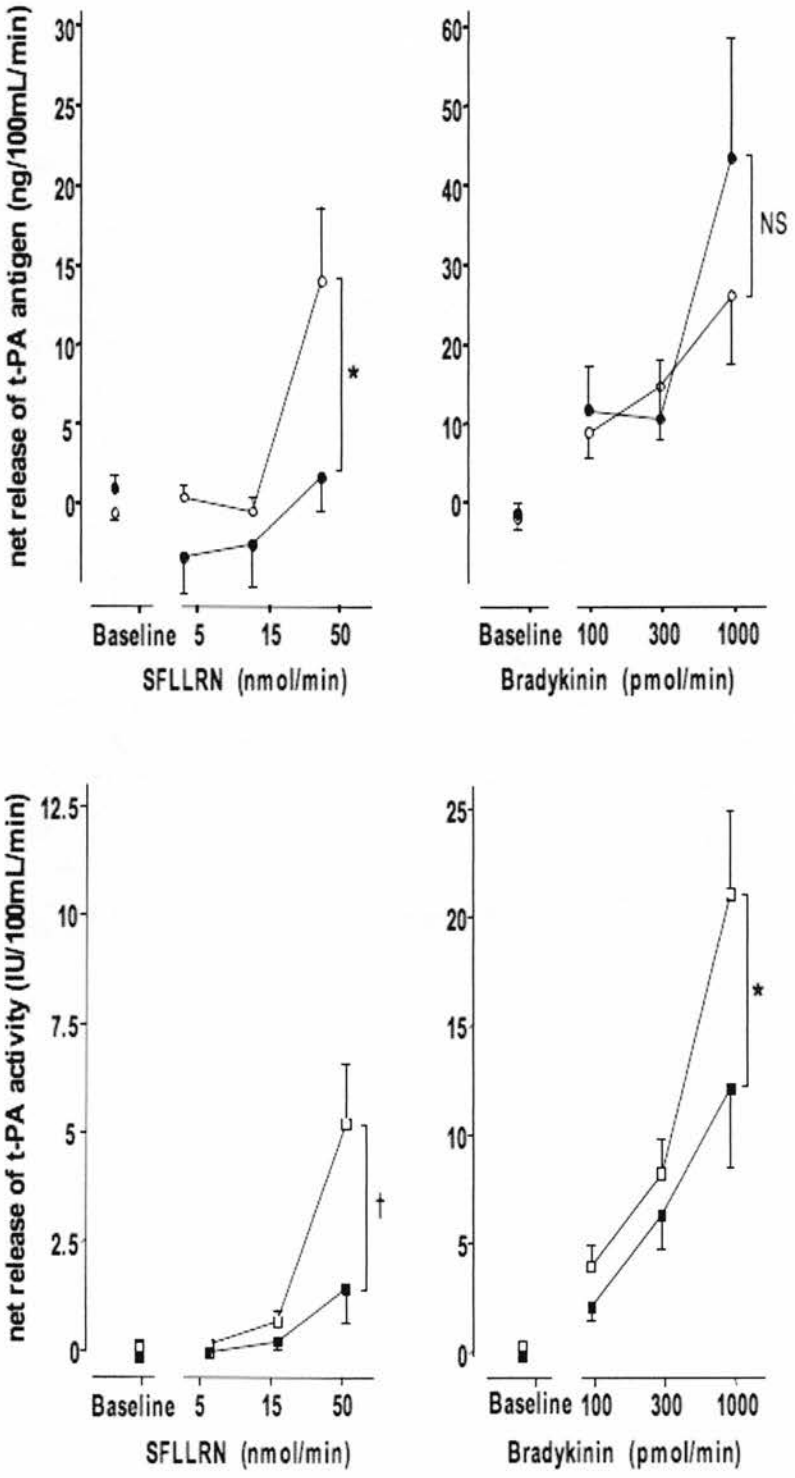


Figure 6.2 *Net release of tissue plasminogen activator (t-PA) in smokers and non-smokers. Net t-PA antigen (circles) and activity (squares) release induced by SFLLRN (top panels) and bradykinin (bottom panels) in smokers (closed symbols) and non-smokers (open symbols).*
**P<0.05, †P=0.005, NS=non-significant;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

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

We have shown, for the first time, 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], we observed no effect of smoking status on endothelium-dependent vasodilatation to bradykinin or endothelium-independent vasodilatation to sodium nitroprusside. One of the important novel observations from our 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 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 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 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* [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% *vs*. 40-50%) [Newby *et al*, 1999; Pretorius *et al*, 2002]. 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 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, recent work has demonstrated a concomitant rise in beta-thromboglobulin suggesting degranulation of platelet alpha granules [Guðmundsdóttir *et al*, 2007]. 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.

We have 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 it 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, 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 results 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

We have previously demonstrated that PAR-1 mediates arterial vasodilatation via two endothelium-dependent mechanisms, namely 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 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 have been demonstrated 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.

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 co-administration 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 or fibrinolytic responses to SFLLRN [Guðmundsdóttir et al, 2006]. 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.

6.6 CONCLUSION

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

CONCLUSION

7.1 SUMMARY

The endothelium is crucial for the control of vascular function and for the maintenance of vascular health. As such, it has been the subject of intense research that has provided the detailed characterisation of some of its vital functions. However, a large proportion of its actions and mediators remain unknown or incompletely described – this is particularly true for EDHF. Consequently, EDHF remains unmanipulated as a therapeutic target despite its involvement in a wide variety of vascular diseases [Feletou and Vanhoutte, 2004]. Furthermore, it has not previously been possible to examine safely the *in vivo* interaction between thrombotic factors and endothelium-derived mediators, including EDHF. The elucidation of the mechanisms underlying this interaction are of great relevance not only for our understanding of atherothrombotic processes but also to ensure the safe and efficient development of novel drugs directed against PAR-1, the principal thrombin receptor in man.

In this thesis, I have assessed thrombotic and endothelium-derived factors in the mediation of vasomotion and endogenous fibrinolysis in man. Specifically, I have examined the role of gap junctions and connexins in the EDHF mechanism and have explored the role of EDHF in the mediation of t-PA release. Using a PAR-1 activating peptide, I have assessed the endothelial mechanisms via which thrombin evokes its vascular effects. Having demonstrated a major interaction between PAR-1 and endothelium-derived factors, I investigated the consequences of cigarette smoking upon vascular responses to PAR-1 activation.

7.2 GAP JUNCTIONS AND EDHF-MEDIATED VASODILATATION

7.2.1 GAP JUNCTION BLOCKADE WITH CONNEXIN MIMETIC PEPTIDES

I have confirmed that EDHF accounts for approximately 50% of bradykinin-induced vasorelaxation in subcutaneous resistance arteries from pregnant women. Previous studies using early putative gap junction blockers suggested a role for gap junctions in the human EDHF mechanism [Kenny *et al*, 2002; Gillham *et al*, 2003; Luksha *et al*, 2004]. However, these early gap junction antagonists have been associated with a variety of non-specific effects and interpretation of those studies should be cautious [Chaytor *et al*, 2000; Matchkov *et al*, 2004].

Using connexin mimetic peptides, I made the first robust examination of the role of gap junctions and their connexin components in EDHF-mediated vasodilatation in human resistance arteries. The specificity of the CMPs has previously been demonstrated [Matchkov *et al*, 2006] and, in my study, they had no effect upon vasorelaxation evoked by endothelium-independent agonists nor did they alter the magnitude of constriction induced by norepinephrine. However, EDHF-mediated vasodilatation was virtually abolished by the addition of a combination of CMPs targeted against the three major vascular connexins (Cx37, Cx40 and Cx43) and provides evidence for a central role of gap junctions in EDHF-mediated vasodilatation in these human arteries. Incubation of vessels with individual CMPs allowed the assessment of the role of single connexin subtypes in the mediation of EDHF. Intriguingly, incubation with a single CMP targeting Cx43 virtually

abolished EDHF-mediated vasodilatation whilst incubation with peptides targeted against Cx37 and Cx40 was without effect. Therefore, despite immunohistochemical evidence for the expression of the three principal vascular connexins, I have demonstrated that only Cx43 is required for the mediation of EDHF in these human vessels.

7.2.2 Cx43 and the EDHF mechanism

The caesarean section incision margin allows ready access to human resistance vessels that have frequently been used to further our understanding of human physiology and, when present, the pathophysiology of pre-eclampsia [Kenny *et al*, 2002; Gillham *et al*, 2003]. It should be noted that normal pregnancy is associated with a 50% rise in circulating blood volume that is accommodated, in part, by an up-regulation of EDHF. Similarly, Cx43 expression is increased under the influence of oestrogen [Liu *et al*, 2002; Nawate *et al*, 2005]. It could be argued that these physiologic changes render it difficult to extrapolate my findings to men and to non-pregnant women. Conversely, the observation that EDHF activity and Cx43 are up-regulated simultaneously may represent further evidence of a central role for Cx43 in EDHF-mediated vasodilatation.

Connexin 43 expression is consistently labile [Gabriels and Paul, 1998; Depaola *et al*, 1999; Inai *et al*, 2004; Figueroa *et al*, 2006] and its expression and function are particularly susceptible to alteration by pathophysiologic processes [Yeh *et al*, 1997; Haefliger *et al*, 2006; Yeh *et al*, 2006]. Whilst similar studies in a variety of animal models and in a variety of vascular beds have demonstrated roles for Cx37 and Cx40

in addition to Cx43 [De Vriese *et al*, 2002; Chaytor *et al*, 2003; Ujiie *et al*, 2003; Chaytor *et al*, 2005], it remains conceivable that Cx43 acts as the principal regulatory component of gap junction functionality in man. Therefore, understanding the role of Cx43 in the EDHF mechanism is of particular interest not only to further our understanding of physiology but also to identify it as a potential therapeutic target. Novel strategies to improve endothelial function via the augmentation of EDHF could herald a breakthrough in the treatment of a variety of vascular diseases. However, their potential would be greatest in the treatment of conditions specifically associated with impaired EDHF activity, such as pre-eclampsia [Kenny *et al*, 2002], type I diabetes mellitus [Fukao *et al*, 1997; Wigg *et al*, 2001; Angulo *et al*, 2003] and possibly hypertension [Fujii *et al*, 1993].

7.2.3 Potentiation of communication via connexin 43

Rotigaptide displays antiarrhythmic properties [Xing *et al*, 2003; Guerra *et al*, 2006; Hennan *et al*, 2006; Shiroshita-Takeshita *et al*, 2007] and acts by augmenting communication via Cx43 [Axelsen *et al*, 2006; Clarke *et al*, 2006]. I obtained custom synthesised rotigaptide and its biological activity was confirmed in an *ex vivo* electrophysiological mapping system. In this model, it increased transmural cardiac conduction velocity at a concentration similar to that predicted to be achieved in my subsequent clinical study.

Using rotigaptide, I made the first assessment of the vascular effects of augmentation of communication via Cx43 in man. Having demonstrated that Cx43 is required for EDHF-mediated vasodilatation in human resistance arteries *in vitro,* it was

hypothesised that rotigaptide would enhance EDHF-mediated vasodilatation by its effects upon Cx43. However, I have shown that intra-arterial rotigaptide does not alter basal vascular tone in the forearm arterial circulation of healthy volunteers. Similarly, continuous intra-arterial administration of rotigaptide does not augment endothelium-dependent agonist-induced vasodilatation, nor does it affect endothelium-independent responses to sodium nitroprusside. Furthermore, these findings remained consistent when the EDHF component of endothelium-dependent vasodilatation was isolated by the inhibition of NO and PGI_2 synthesis. Therefore, it can be concluded that augmentation of communication via Cx43 with rotigaptide does not enhance endothelial function in the forearm circulation of healthy volunteers.

These findings should not be extended to imply that Cx43 is unimportant in the human EDHF mechanism. In the healthy circulation, the open-state probability of Cx43 is likely to be high and efforts directed to further opening of Cx43 may be futile. However, the open-state probability of Cx43 is depressed by acidosis [Ek-Vitorín *et al*, 1996] and rotigaptide's antiarrhythmic activity is more potent in conditions of metabolic stress [Eloff *et al*, 2003]. *In vitro,* the open-state probability of Cx43 is depressed by hyperglycaemia [Inoguchi *et al*, 2001; Fernandes *et al*, 2004], lipopolysaccharide [Lidington *et al*, 2002] and during reperfusion after ischaemia [Zhang *et al*, 1999]. It remains possible, although speculative at this point, that rotigaptide augments EDHF-mediated vasodilatation in conditions of metabolic stress including during hyperglycaemia or immediately following ischaemia. Furthermore, it is unknown whether the enhancement of communication via Cx43 is

an appropriate strategy for the treatment of endothelial dysfunction associated with a wider range of pathophysiologic processes.

7.3 ENDOTHELIAL MEDIATORS OF VASCULAR PAR-1 RESPONSES

Thrombin is central to the pathophysiology of atherothrombosis. However, its potent stimulatory effects upon the coagulation cascade have previously made it impossible to safely examine its vascular effects *in vivo* in man. However, PAR-1 has been identified as thrombin's major cellular receptor [Vu *et al*, 1991] and the therapeutic potential of PAR-1 antagonists is currently being assessed in clinical trials [Husted, 2007]. My colleagues have previously demonstrated that SFLLRN, a synthetic PAR-1 activating peptide, causes venoconstriction, arterial vasodilatation and t-PA release *in vivo* in man [Guðmundsdóttir *et al*, 2006]. In this thesis, I have assessed the role of the endothelium in the mediation of these effects.

SFLLRN caused constriction of the dorsal hand vein in both the presence and absence of local endothelium. Therefore, it can be concluded that venoconstriction evoked by PAR-1 activation is mediated by smooth muscle PAR-1 receptors and not via the release of endothelium-derived vasoconstrictors such as endothelin or angiotensin II.

In contrast, I have demonstrated that PAR-1 activation causes arterial vasodilatation via an endothelium-dependent mechanism. I have shown that PAR-1 activation does not evoke a discernible PGI_2-dependent vasodilator response. However, using TEA

and 'the nitric oxide clamp' I have demonstrated major contributions from both EDHF and NO, respectively. These findings are consistent with data from *in vitro* studies [Hamilton *et al*, 1998; Hamilton *et al*, 2001; Kawabata *et al*, 2004a; Kawabata *et al*, 2004b] and illustrate the importance of an intact endothelium for the arterial vasculature to respond to the presence of thrombin. In the presence of developing thrombus, arterial vasodilatation may represent an important safety mechanism for the dispersal of thrombus whilst the associated endothelial release of fibrinolytic factors causes its dissolution [Guðmundsdóttir *et al*, 2006].

7.4 ENDOTHELIUM-DERIVED HYPERPOLARISING FACTOR AND T-PA RELEASE

It has previously been suggested that the endothelial release of t-PA may be evoked via EDHF [Brown *et al*, 2000; Hrafnkelsdottir *et al*, 2001; Oliver *et al*, 2005] but direct *in vivo* testing of this hypothesis had not been performed. The findings described in Chapter 5 demonstrate that inhibition of NO activity augments t-PA release evoked by PAR-1 activation and others have shown that bradykinin-induced t-PA release is increased during concurrent NO inhibition [Smith *et al*, 2003]. In isolation, these findings could be extrapolated to argue for a role of EDHF in t-PA release. Indeed, the vasomotor actions of EDHF have consistently been shown to be reciprocal to those of NO [Bauersachs *et al*, 1996] and, if EDHF is associated with t-PA release, a similar augmented fibrinolytic response might be expected in the absence of NO. However, I have shown that whilst TEA inhibits the EDHF component of PAR-1 evoked vasodilatation, this is not accompanied by a similar

alteration in t-PA release. Furthermore, bradykinin-induced t-PA release was correspondingly unaffected by the presence of TEA. It can be concluded that, in the healthy human forearm circulation, EDHF does not contribute to the mechanism for endothelial t-PA release. This is consistent with the observation that t-PA release occurs in response to inflammatory mediators including TNF-α in the absence of vasodilatation [Chia *et al*, 2003] and that t-PA is released by norepinephrine which causes vasoconstriction [Jern *et al*, 1994].

These findings do not, however, exclude the possibility that factors involved in the downstream signalling mechanism of EDHF are shared by the t-PA release mechanism. Activation of calcium-activated potassium channels is one of the earliest events in the generation of hyperpolarisation and others have demonstrated that K_{Ca} inhibition with TEA does not affect thrombin-induced endothelial t-PA release *in vitro* [Muldowney *et al*, 2007]. However, in that study, the elimination of isoforms of EET inhibited t-PA release [Muldowney *et al*, 2007] and others have demonstrated that application of 11,12-EET increases tissue t-PA expression in cultured endothelial cells [Node *et al*, 2001]. It is of note that there is also a substantial body of evidence to suggest that these cytochrome P450 metabolites of arachidonic acid are involved in the mediation of EDHF activity [Fisslthaler *et al*, 1999; Halcox *et al*, 2001; Archer *et al*, 2003; Feletou and Vanhoutte, 2006a]. An *in vivo* examination of the role of EETs in t-PA release has not been made.

Using rotigaptide, I assessed the effect of augmentation of communication via Cx43 upon endothelial t-PA release and demonstrated that rotigaptide does not enhance

bradykinin-induced endogenous fibrinolysis in healthy volunteers. Whether it has any effect upon t-PA release in patients with impaired endothelial function remains unknown.

7.5 CIGARETTE SMOKING AND ENDOTHELIAL PAR-1 RESPONSES

Having demonstrated that PAR-1 activation mediates arterial vasodilatation and the release of endogenous fibrinolytic factors via endothelium-dependent mechanisms, I hypothesised that cigarette smoking would be associated with endothelial dysfunction and, consequently, reduced vascular PAR-1 responses. In the forearm arterial circulation, I demonstrated that PAR-1 mediated arterial vasodilatation and t-PA release were markedly impaired by cigarette smoking. Indeed, PAR-1 induced t-PA antigen release was almost abolished by long-term cigarette smoking. Similarly, PAR-1 evoked t-PA activity was substantially less in smokers than in age-matched non-smokers. The combination of impaired PAR-1 mediated arterial vasodilatation and t-PA release seen in smokers would favour clot expansion and vessel occlusion with clinically relevant sequelae including myocardial infarction and stroke. This important shift in the fine balance between clot propagation and dissolution is likely to contribute to the elevated atherothrombotic risk associated with cigarette smoking.

The major advance represented by this study is the assessment of endothelial responses to PAR-1 activation. By examining responses to the major cellular receptor for thrombin, the principal mediator of atherothrombosis, I have been able to make the most pathophysiologically relevant assessment of endothelial function

thus far. Previous studies have demonstrated impaired substance P- and bradykinin-induced vasodilatation and fibrinolytic capacity in the peripheral [Newby *et al*, 1999; Pretorius *et al*, 2002] and coronary [Newby *et al*, 2001; Takashima *et al*, 2007] circulation of smokers. Substance P and bradykinin are useful tools for the assessment of endothelial function but, particularly in the case of substance P, their direct pathophysiological relevance to atherothrombosis is substantially less than that of thrombin and PAR-1 activation.

I have demonstrated that cigarette smoking is associated with impaired PAR-1 mediated arterial vasodilatation but, in the smokers studied here, this occurred in the absence of a discernible attenuation of the vasomotor response evoked by bradykinin. Furthermore, the magnitude of the reduction in t-PA release was substantially greater for PAR-1 evoked responses than for responses to substance P [Newby *et al*, 1999] and bradykinin [Pretorius *et al,* 2002]. Therefore, it can be argued that the assessment of vascular responses to PAR-1 activation is not only pathophysiologically relevant, but also sensitive for the detection of endothelial dysfunction.

It has previously been demonstrated that cigarette smoking impairs endothelial function primarily via a diminution of NO bioavailability [Ambrose and Barua, 2004]. However, the relative impairment of NO and EDHF activity caused by cigarette smoking has never been assessed *in vivo* in man. In this thesis, I have demonstrated that cigarette smoking is associated with reduced PAR-1 evoked t-PA release and have also shown that PAR-1 activation mediates t-PA release via a NO-,

PGI_2- and K_{Ca}-independent mechanism. Taken together, these findings highlight that the endothelial dysfunction arising from exposure to the constituents of cigarette smoke is complex and constitutes more than an isolated deficiency of NO bioavailability. It remains unknown whether cigarette smoking causes a decrease in the bioavailability of mediators that could conceivably be common to both the EDHF and t-PA release mechanisms, such as EETs. Furthermore, the effect of smoking upon myoendothelial communication via gap junctions remains unknown.

7.6 FUTURE DIRECTIONS

7.6.1 EDHF AND GAP JUNCTIONS

Further understanding of the role of gap junctions and connexins in the EDHF mechanism is required not only to unravel human physiologic mechanisms, but also to assess their potential as therapeutic targets in a wide variety of disease states. Although not specifically designed to do so, agents such as rotigaptide have the potential to interact with the EDHF mechanism. Drugs of this class may not only prove to be useful tools for the assessment of gap junctions and EDHF but may also have therapeutic potential that is distinct from their intended utility as antiarrhythmic agents.

7.6.2 EDHF, GAP JUNCTIONS AND CONGESTIVE HEART FAILURE

Congestive cardiac failure is common in western society, is associated with significant morbidity and carries a prognosis that is worse than that of many cancers [Mosterd and Hoes, 2007]. There is considerable evidence that EDHF becomes up-

regulated and partially compensates for impaired NO bioavailability associated with heart failure [Malmsjo *et al*, 1999; Katz and Krum, 2001; Ueda *et al*, 2005].

In a series of wire-myography studies similar to those described in Chapter 3, the CMPs could be used to examine the role of gap junctions and connexins in subcutaneous resistance arteries from patients with chronic heart failure and from age- and sex-matched controls. Indeed, fat biopsies could be obtained from the implant site of patients with a history of congestive cardiac failure undergoing pacemaker and/or implantable defibrillator insertion. The recent increase in device implantation in patients with congestive cardiac failure provides a large population suitable for inclusion in this type of study. Furthermore, a large proportion of patients undergo device insertion for non-ischaemic indications and have no history of congestive cardiac failure. This group would, therefore, be ideal for the provision of biopsies from age- and sex-matched control volunteers.

By examining the proportional contribution of EDHF to endothelium-dependent vasodilatation in the presence and absence of heart failure, this study would allow the first direct assessment of the effect of heart failure upon EDHF-mediated responses in man. Furthermore, the role of gap junctions in the mediation of the EDHF response could be assessed by using a combination of the three CMPs employed in Chapter 3. Additionally, by assessing the effect of the addition of individual peptides, the relative contributions of the three principal vascular connexins could be assessed. It remains unknown whether Cx43 is the predominant functional connexin isoform in the non-pregnant state and, whilst it tends to be the most labile, it is

unknown whether its isolated up-regulation is responsible for any increase in EDHF activity associated with heart failure.

7.6.3 PRE-ECLAMPSIA AND THE AUGMENTATION OF COMMUNICATION VIA CONNEXIN 43

Pre-eclampsia is defined as the association of new onset hypertension and proteinuria during pregnancy. It affects 3-5% of all pregnancies and is associated with significant morbidity and mortality for both mother and baby [Meis *et al*, 1998]. Pre-eclampsia frequently causes intra-uterine growth restriction and these low birthweight babies are at increased risk of developing cardiovascular disease in adulthood [Ozanne *et al*, 2004]. In the United Kingdom, hypertensive disorders of pregnancy are the second most common cause of maternal mortality and epidemiologic data suggest that pre-eclampsia increases the mother's future risk of hypertension, coronary artery disease and stroke [Wilson *et al*, 2003].

As noted previously, normal pregnancy is associated with vascular adaptations that permit a large rise in blood volume and cardiac output. Increased NO, and particularly EDHF, activity provide a decrease in peripheral vascular resistance to allow these changes to occur without a concomitant rise in blood pressure [Gillham *et al*, 2003]. However, impaired EDHF-mediated vasodilatation has been observed in resistance vessels from women with pre-eclampsia and is proposed as a major pathogenic mechanism [Kenny *et al*, 2002; Gillham *et al*, 2003].

Rotigpatide could be used to make the first direct assessment of the role of Cx43 in the pathophysiology of pre-eclampsia. Subcutaneous resistance arteries from the caesarean section incision margin of women with pre-eclampsia and normotensive pregnant women could be obtained and vascular responses examined using wire-myography. By examining the vascular effects of the addition of rotigaptide, the functional role of Cx43 in the impairment of EDHF activity associated with pre-eclampsia could be assessed. Whilst the findings demonstrated in this thesis do not demonstrate an effect of rotigaptide upon EDHF activity in healthy subjects, its effects may become apparent in conditions of impaired EDHF activity when the open-state probability of Cx43 is lower. Therefore, this experiment would allow the testing of the hypothesis that rotigaptide restores EDHF activity in resistance vessels from women with pre-eclampsia. In addition, immunohistochemistry would allow an examination of the effects of pre-eclampsia upon the expression of the vascular connexins.

A study of this type would provide important mechanistic insights to pre-eclampsia. Furthermore, it would have the potential to highlight gap junctions as a drug target not only in the treatment and prevention of pre-eclampsia, but also other vascular disorders associated with impaired EDHF function, including type I diabetes mellitus.

7.6.4 The mechanism of endothelial t-PA release

Detailed understanding of the pathway and mediators via which the endothelium releases t-PA is still lacking. However, the endothelial capacity for t-PA release is

now accepted as an important marker of both endothelial function and future cardiovascular risk [Oliver *et al*, 2005; Robinson *et al*, 2007]. Indeed, it is recognised that enhanced endothelial t-PA release makes a significant contribution to the anti-ischaemic benefits afforded by angiotensin-converting enzyme inhibition [Witherow *et al*, 2002; Pretorius *et al*, 2003]. The identification of mediators of endothelial t-PA release would have the potential to guide the development of novel drugs designed specifically to enhance appropriate endogenous fibrinolysis in patients at risk of atherothrombotic disorders, such as myocardial infarction and stroke.

As discussed, *in vitro* evidence suggests that EETs are involved in the mediation of endothelial t-PA release [Node *et al*, 2001; Muldowney *et al*, 2007] but this has not been demonstrated *in vivo*. Cytochrome P450 antagonists, such as miconazole and sulphaphenazole, inhibit the *in vivo* synthesis of EETs. In the forearm circulation, these antagonists attenuate EDHF-mediated vasodilatation when NO and PGI_2 synthesis are inhibited [Halcox *et al*, 2001; Taddei *et al*, 2006]. However, their effect upon stimulated endothelial t-PA release has not been examined and a study to assess the role of EETs in the *in vivo* mechanism for t-PA release is overdue. In the forearm model, either of these antagonists could be used to provide useful information about the regulation of basal t-PA release and the mechanism of t-PA release stimulated by PAR-1 activation or bradykinin, for example.

7.6.5 PROTEASE-ACTIVATED RECEPTOR TYPE 1 ANTAGONISTS

The clinical development of the PAR-1 antagonists has been driven primarily by the desire to create a novel anti-platelet agent that is not associated with the

anticoagulant and bleeding effects seen with direct thrombin inhibitors [Chackalamannil, 2006]. However, the findings described in this thesis highlight the important interaction that these drugs may have with the endothelium. In addition to their useful anti-platelet effects, a thorough examination of their vascular effects is warranted to ensure that any therapeutic gains are not offset, or even reversed, by their potential to alter vascular tone and endogenous fibrinolysis.

Whilst I have demonstrated that PAR-1 activation causes local vasodilatation via the release of endothelium-derived factors, the role of PAR-1 in the maintenance of basal vascular tone remains unknown. By examining the effects of an intra-arterial PAR-1 antagonist in the forearm, important information regarding the role of PAR-1 in the maintenance of basal vascular tone and endogenous fibrinolysis would be gained. In the meantime, clinical trials should be designed and interpreted to take into account the potential for systemic PAR-1 antagonism to be associated with potentially harmful endothelial effects.

7.7 CONCLUDING REMARKS

The potential for the therapeutic modulation of EDHF activity relies upon a more detailed understanding of its mechanism. In conjunction with the requirement for this mechanistic knowledge, there is a need to further clarify its dynamic role in disease states in man. Whilst its up-regulation has been described frequently as a protective back-up mechanism to compensate for impaired NO bioavailability, this finding is not universal. Indeed, specific impairment of EDHF activity has been described in a

variety of conditions. However, a major barrier to the conclusive understanding of these interactions includes the marked variations in EDHF activity and its interaction with co-morbid conditions in different species. From here, research needs to focus upon its assessment in man in order to come to relevant conclusions to guide drug development. By making this thorough assessment, the therapeutic utility of gap junction manipulation should become more apparent and efforts to improve intercellular communication may be targeted to appropriate disease states.

Increased understanding of endothelium-derived factors, their mechanisms of action and their dynamic interactions will also further highlight the potential for an array of drugs to make unintentional interactions with the endothelium. Indeed, a wide variety of drugs may provoke a range of endothelial effects that are distinct from their intended therapeutic application. Whilst some of these effects may fortuitously be beneficial, it remains vital to be alert to the risk of harmful side-effects as a result of altered endothelial function. Indeed, the beneficial anti-platelet effects of PAR-1 antagonism may be offset by their potential to decrease endothelial PAR-1 responses. As discussed, PAR-1 mediated local arterial vasodilatation and t-PA release may represent an important protective mechanism in the presence of developing arterial thrombus. It is conceivable that loss of this mechanism for clot dispersal and dissolution could result in a paradoxical acceleration of thrombus growth and an increased risk of clinically relevant vessel occlusion. These deleterious effects may be further exaggerated in patients with pre-existing endothelial dysfunction in whom PAR-1 mediated vasodilatation and t-PA release are already impaired. Of course,

these individuals are representative of the population in whom PAR-1 antagonists are likely to be used for their anti-platelet effects.

The requirement for an efficacious oral anti-platelet agent without a high risk of attendant bleeding complications would represent a breakthrough in the treatment of atherothrombotic disorders. Therefore, the clinical development of PAR-1 antagonists continues with cautious optimism. Their therapeutic utility will depend upon the net effect of their beneficial anti-platelet effects balanced against potentially deleterious effects upon the endothelium. These opposing effects should not be seen as an absolute barrier to their development but they further illustrate the complexities of endothelial function and the dynamic nature of its mediators. As long as drug development pays credence to these interactions and is mindful of the vital roles and the power of endothelial mediators, the endothelium should continue to yield some of the most important targets for the improvement of vascular health.

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APPENDIX

ABBREVIATIONS

ANOVA	Analysis of variance
AP	Action potentials
AT	Activation time
ATP	Adenine triphosphate
BK_{Ca}	Large calcium-activated potassium channels
Ca^{2+}	Calcium ions
cAMP	Cyclic adenosine monophosphate
CMPs	Connexin mimetic peptides
Cx	Connexin
EDHF	Endothelium-derived hyperpolarising factor
EET	Epoxyeicosatrienoic acid
eNOS	Endothelial nitric oxide synthase
FBF	Forearm blood flow
Hct	Haematocrit
ICAM-1	Intercellular adhesion molecule-1
IK_{Ca}	Intermediate calcium-activated potassium channels
K^+	Potassium ions
K_{Ca}	Calcium-activated potassium channels
KPSS	High potassium physiological saline solutation
L-NAME	N^{ω}-nitro-L-arginine-methyl ester
L-NMMA	L-N^G-monomethyl arginine
LVDT	Linear variable differential transducer

NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor type 1
PAR-1	Protease-activated receptor type 1
PGI_2	Prostacyclin
PSS	Physiological salt solution
SK_{Ca}	Small calcium-activated potassium channels
SNP	Sodium nitroprusside
TEA	Tetraethylammonium ion
TNF-α	Tumour necrosis factor-alpha
t-PA	Tissue-type plasminogen activator
UTP	Uridine triphosphate
VCAM-1	Vascular cell adhesion molecule-1
vWF	von Willebrand factor

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Lang NN, Myles RC, Burton FL, Hall DP, Chin YZ, Boon NA, Newby DE. The Vascular Effects of Rotigaptide *In Vivo* in Man. *Biochem Pharmacol.* In press.

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Connexin 43 mediates endothelium-derived hyperpolarizing factor-induced vasodilatation in subcutaneous resistance arteries from healthy pregnant women

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Lang NN, Luksha L, Newby DE, Kublickiene K. Connexin 43 mediates endothelium-derived hyperpolarizing factor-induced vasodilatation in subcutaneous resistance arteries from healthy pregnant women. *Am J Physiol Heart Circ Physiol* 292: H1026–H1032, 2007. First published November 3, 2006; doi:10.1152/ajpheart.00797.2006.—The role of gap junctions in endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation of human arteries was assessed using connexin mimetic peptides (CMPs) designated ^{37,43}Gap27, ^{40}Gap27, and ^{43}Gap26 according to homology with the major vascular connexins (Cx37, Cx40, and Cx43). Resistance arteries were obtained from subcutaneous fat biopsies of healthy pregnant women undergoing elective cesarean section. Endothelium-dependent vasodilatation to bradykinin (BK) was assessed using wire myography. N^{ω}-nitro-L-arginine methyl ester (L-NAME) and indomethacin (nitric oxide synthase and cyclooxygenase inhibitors, respectively) attenuated maximal relaxation to BK (R_{max}) by ~50%. Coincubation with L-NAME, indomethacin, and the combined CMPs (^{37,43}Gap27, ^{40}Gap27, and ^{43}Gap26) almost abolished relaxation to BK (R_{max} = 12.2 ± 3.7%). In arteries incubated with L-NAME and indomethacin, the addition of either ^{37,43}Gap27 or ^{40}Gap27 had no significant effect on R_{max}, whereas ^{43}Gap26 caused marked inhibition (R_{max} = 21 ± 6.4%, P = 0.005 vs. L-NAME plus indomethacin alone) that was similar to that of the triple combination. Endothelium-independent vasorelaxation was unaffected by CMPs, L-NAME, or indomethacin. Immunohistochemistry demonstrated Cx37, Cx40, and Cx43 expression in the endothelium and vascular smooth muscle. In pregnant women, EDHF-mediated vasorelaxation of subcutaneous resistance arteries is dependent on Cx43 and gap junctions.

connexin mimetic peptides; gap junctions; microcirculation; bradykinin

NITRIC OXIDE (NO), the original endothelium-derived relaxing factor, and prostacyclin (PGI_2) have now been well characterized. The elucidation of their roles in vascular physiology and pathophysiology has been fundamental to recent advances in the treatment and prevention of many cardiovascular diseases.

Evidence points to the existence of a third powerful vasodilator called endothelium-derived hyperpolarizing factor (EDHF) (2, 12, 27). The contribution of EDHF to endothelium-dependent vasorelaxation is greatest in small resistance arteries (1) and, as such, is ideally suited to the control of systemic blood pressure and local tissue perfusion. As well as its involvement in normal physiological responses, EDHF is believed to play an important role in vascular pathophysiology. It has been suggested to act as a "back-up" mechanism in conditions associated with decreased NO bioavailability, such as heart failure (13, 18). Furthermore, failure of EDHF to compensate in this way has been implicated in the pathogenesis of preeclampsia (14).

Despite intensive research, the exact nature of EDHF and its mechanism of action remain unclear. This lack of understanding has precluded its manipulation as a therapeutic target (10). Multiple candidates have been proposed, but none have been confirmed as a unifying agent constituting EDHF. As such, the term "endothelium-derived hyperpolarizing factor" may be misleading, and EDHF may represent a mechanism rather than a specific factor per se.

The case for gap junctions as a central component of the EDHF mechanism has strengthened (12, 28). These aqueous pores facilitate the transfer of either small hydrophilic molecules or ionic charge for the conduction of hyperpolarization from the endothelium to smooth muscle and from smooth muscle cell to smooth muscle cell. Gap junctions are composed of two hemi-channels comprising six connexin (Cx) subunits each. Cx37, Cx40, and Cx43 are particularly associated with mammalian endothelium and vascular smooth muscle (12).

Diverse agents have been employed as putative gap junction blockers, but their other nonspecific, EDHF-independent effects have limited their use. In contrast, the connexin mimetic peptides (CMPs) are highly selective and specific (12, 20). These short synthetic peptides correspond to conserved amino acid sequences in the first (Gap 26) and second (Gap 27) extracellular loops of Cx37, Cx40, and Cx43 and cause disruption of connexin function (5).

Pregnancy is associated with a decrease in peripheral vascular resistance and enhanced endothelium-dependent vasodilatation (11). We have previously reported that EDHF accounts for ~50% of endothelium-dependent vasodilatation in these arteries. Furthermore, using the putative gap junction blocker 18α-glycyrrhetinic acid, our group identified a potential role of gap junctions in EDHF responses (17). In this study we tested the hypothesis that gap junction blockade with CMPs inhibits EDHF-mediated vasorelaxation in resistance vessels from pregnant women. Furthermore, we examined the expression of the three main vascular connexin subtypes in these vessels. By using CMPs, the current study represents the first assessment

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of the functional role of gap junctions and their connexin components in EDHF-mediated relaxation in the human vasculature.

MATERIALS AND METHODS

Biopsy collection and vessel preparation. The investigation was undertaken with the approval of the local research ethics committee, with the written informed consent of each patient, and in accordance with the principles outlined in the Declaration of Helsinki. Subcutaneous fat biopsies were obtained from healthy women undergoing elective cesarean section. Women with a history of diabetes mellitus, hypertension, or any other significant past medical history were excluded from participation. Women with abnormal renal or hepatic function also were excluded. Biopsies were taken from the incision margin and immediately placed in cold physiological salt solution (PSS: 119 mmol/l NaCl, 4.7 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.17 mmol/l $MgSO_4$, 25 mmol/l $NaHCO_3$, 1.18 mmol/l KH_2PO_4, 0.026 mmol/l EDTA, and 5.5 mmol/l glucose).

Resistance arteries were dissected free of surrounding fat and connective tissue and cut into segments ~2 mm long. All arteries were kept in PSS at 4°C and used on the day of biopsy. Arteries were mounted in the organ baths of a four-channel wire myograph (multimyograph model 610; Danish Myo Technology, Aarhus, Denmark) as described previously (17). Each organ bath contained warmed (37°C) PSS and was continuously bubbled with 5% carbon dioxide-95% oxygen. After a 30-min equilibration period, a passive circumference-tension curve was created for each segment to set optimum resting tension. This resting tension is calculated to simulate an in vivo transmural pressure of 100 mmHg. Arteries were then set at 90% of this tension to enable optimal contractile conditions, as described previously (17, 22). This semiautomated procedure also allows the calculation of arterial diameter. Calibration and data processing were performed using Myodac software (version 2.1; Danish Myo Technology) on a personal computer. All solutions were refreshed every 30 min.

Constriction was elicited using norepinephrine (NE; 3 μmol/l). Endothelium-dependent vasodilatation was assessed by the addition of a single dose of bradykinin (BK; 1 μmol/l) to each chamber. Arteries that did not achieve at least 60% vasorelaxation to BK were excluded from the study.

Experimental protocols. Arteries were incubated for 40 min in either PSS alone or with the nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 300 μmol/l) and the cyclooxygenase (COX) inhibitor indomethacin (10 μmol/l) to block the production of NO and PGI_2, respectively. Arteries were then preconstricted with NE (3 μmol/l), and the concentration-response curve for BK (0.001–3 μmol/l) was assessed. Arteries then underwent 90 min of incubation with CMPs with or without L-NAME and indomethacin as detailed below. The concentration-response curve to BK was then reassessed. At the end of each experiment, endothelium-independent vasorelaxation was assessed in response to sodium nitroprusside (SNP; 0.1 mmol/l). The doses of the CMPs used were based on prior published work (4, 12).

Gap junction inhibition with and without NOS/COX inhibition. Arteries initially incubated in PSS alone were incubated with PSS plus the combined CMPs ^{37,43}Gap27 (300 μmol/l), ^{40}Gap27 (300 μmol/l), and ^{43}Gap26 (300 μmol/l). The contribution of gap junctions to EDHF-mediated vasorelaxation with concurrent NOS/COX inhibition was assessed by preincubating with L-NAME and indomethacin before administering the combined CMPs (as above) in the continued presence of L-NAME and indomethacin.

Contribution of connexin subtypes to EDHF-mediated relaxation. Arteries preincubated with L-NAME and indomethacin alone were incubated with L-NAME and indomethacin plus *1*) ^{37,43}Gap27 plus ^{40}Gap27 (450 μmol/l each), *2*) ^{37,43}Gap27 (900 μmol/l), *3*) ^{40}Gap27 (900 μmol/l), or *4*) ^{43}Gap26 (900 μmol/l). Total peptide concentration in each experiment was kept constant at 900 μmol/l.

Effects of ^{43}Gap26 alone on endothelium-dependent and -independent vasorelaxation. Arteries initially incubated in PSS alone were incubated with ^{43}Gap26 (900 μmol/l) for 90 min. A concentration-response curve to BK was constructed (as above) before and after ^{43}Gap26. In further experiments, a concentration-response curve was constructed for either SNP (0.001–1 μmol/l) or the endothelium-independent, ATP-sensitive potassium channel opener pinacidil (0.001–1 μmol/l) before and after incubation with ^{43}Gap26 (again, without L-NAME and indomethacin).

Immunohistochemistry. Freshly isolated artery segments were cryopreserved in optimal cutting temperature (OCT) compound cooled by liquid nitrogen. Transverse 10-μm cryosections were prepared and mounted onto slides, air-dried, and stored at −20°C. Sections were immunostained with polyclonal rabbit antibodies against mouse connexins [Cx37 and Cx40 (1:50 dilution) or Cx43 (1:100 dilution) at 4°C overnight (Zymed Laboratories, San Francisco, CA)] (15, 23). Negative control sections were incubated with nonimmune goat IgG (SDS, Falkenberg, Sweden). All slides were counterstained with hematoxylin, dehydrated, and mounted with Pertex (Histolab, Gothenburg, Sweden).

Drugs and chemicals. All drugs and chemicals except the CMPs and antibodies were obtained from Sigma (St. Louis, MO). The CMPs ^{37,43}Gap27 (SRPTEKTIFII), ^{40}Gap27 (SRPTEKNVFIV), and ^{43}Gap26 (VCYDKSFPISHVR) were purchased from American Peptide (Sunnyvale, CA). All drugs except indomethacin and L-NAME were dissolved in PSS before every experiment. Indomethacin was dissolved in pure ethanol. L-NAME was dissolved in distilled water.

Data analysis. All data are expressed as means ± SE. Maximum vasorelaxation (R_{max}) is expressed as the percentage return to basal tone produced by the highest concentration of BK (3 μmol/l) after NE preconstriction. Analysis of variance (ANOVA) was used to compare paired BK concentration-response curves before and after incubation with CMPs and between groups. Bonferroni's correction for multiple comparisons was used for the analysis of pooled data. Paired Student's *t*-test was used to compare NE preconstriction before and after incubation with different agents (GraphPad Prism version 4.0). Statistical significance was taken at the 5% level.

RESULTS

Subcutaneous fat biopsy specimens were obtained from 14 pregnant women (5 nulliparous) with a median age of 27 yr (range 25–42 yr) and a median gestational age of 38 wk (range 37–40 wk) undergoing planned cesarean section due to breach presentation ($n = 4$), previous cesarean section ($n = 8$), and psychological reasons ($n = 2$).

A total of 31 arteries dissected from 14 biopsies were included in the experiment. Mean internal diameter of artery segments used was 221 ± 8 μm. Vessels with an internal diameter of 100–300 μm were considered to represent resistance arteries, in line with previous definitions (6). There were no significant differences between artery segments used in each protocol with respect to internal diameter and initial constrictor response to NE (data not shown).

Contribution of EDHF to BK-mediated vasorelaxation. When compared with all arteries incubated in PSS alone ($n = 18$), incubation with L-NAME and indomethacin ($n = 27$) reduced maximal vasorelaxation to BK by ~50% (R_{max}: 90.3 ± 2.2% in PSS alone vs. 48.1 ± 2.6% with L-NAME and indomethacin, $P < 0.0001$). Pilot studies confirmed that BK concentrations of >3 μmol/l failed to further relax vessels incubated in PSS alone.

Gap junction inhibition with the combined CMPs [^{37,43}Gap27 plus ^{40}Gap27 plus ^{43}Gap26 (300 μmol/l each)] substantially reduced maximal relaxation to BK (R_{max}: 46.6 ± 2.8% with combined CMPs vs. 89.4 ± 3.5% in PSS alone, P = 0.023, n = 6). The inhibition of gap junction-mediated communication with combined CMPs decreased maximal BK-induced vasorelaxation to an extent similar to that of L-NAME plus indomethacin alone (Fig. 1).

Combined NOS, COX, and gap junction inhibition with the combined CMPs plus L-NAME and indomethacin almost abolished the vasorelaxation to BK (R_{max}: 12.2 ± 3.7% with combined CMPs plus L-NAME and indomethacin vs. 49.0 ± 5.1% with L-NAME and indomethacin, P = 0.0004, n = 6; Fig. 1).

Contribution of different connexin subtypes to EDHF-mediated vasorelaxation. Incubation with ^{37,43}Gap27 (450 μmol/l) plus ^{40}Gap27 (450 μmol/l) in addition to L-NAME and indomethacin did not reduce maximal vasorelaxation to BK compared with the same vessels incubated in L-NAME and indomethacin alone (R_{max}: 38.2 ± 5.6% with L-NAME and indomethacin plus ^{37,43}Gap27 and ^{40}Gap27 vs. 40.6 ± 6.1% with L-NAME and indomethacin, P = 0.319, n = 5; Fig. 2).

The addition of ^{37,43}Gap27 (900 μmol/l) or ^{40}Gap27 (900 μmol/l) to L-NAME and indomethacin had no effect on maximal BK-induced vasorelaxation compared with the same arteries incubated with L-NAME and indomethacin alone (R_{max}: 43.6 ± 1.4% with ^{37,43}Gap27 plus L-NAME and indomethacin vs. 49.6 ± 4.87% with L-NAME and indomethacin alone, P = 0.39, n = 5; R_{max}: 46.3 ± 5.5% with ^{40}Gap27 plus L-NAME and indomethacin vs. 45.0 ± 2.2% with L-NAME and indomethacin alone, P = 0.37, n = 4; Fig. 2). However, the inhibition of Cx43 with ^{43}Gap26 (900 μmol/l) in combination with L-NAME and indomethacin resulted in a marked decrease in maximal vasorelaxation to BK compared with the same arteries incubated with L-NAME and indomethacin alone (R_{max}: 21.0 ± 6.4% with ^{43}Gap26 plus L-NAME and indomethacin vs. 52.45 ± 8.3% with L-NAME and indomethacin alone, P = 0.0047, n = 5). Maximal BK-induced relaxation after incubation with L-NAME and indomethacin plus ^{43}Gap26 (900 μmol/l) was not different from maximal relaxation after incubation with the three combined CMPs (each at 300 μmol/l) (unpaired data, P = 0.397; Fig. 3).

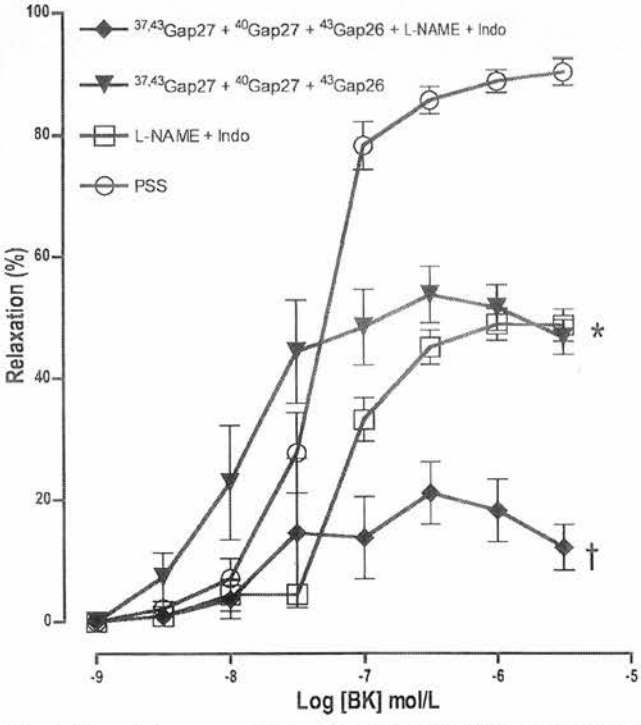

Fig. 1. Concentration-response curves for bradykinin (BK) in subcutaneous arteries incubated in physiological saline solution (PSS; n = 18), N^{ω}-nitro-L-arginine methyl ester (L-NAME) plus indomethacin (n = 25), or the combined connexin mimetic peptides (^{37,43}Gap27, ^{40}Gap27, and ^{43}Gap26 at 300 μmol/l each) with or without L-NAME plus indomethacin (n = 6 for both). Indo, indomethacin. *P < 0.001 vs. PSS. †P < 0.001 vs. L-NAME and indomethacin alone.

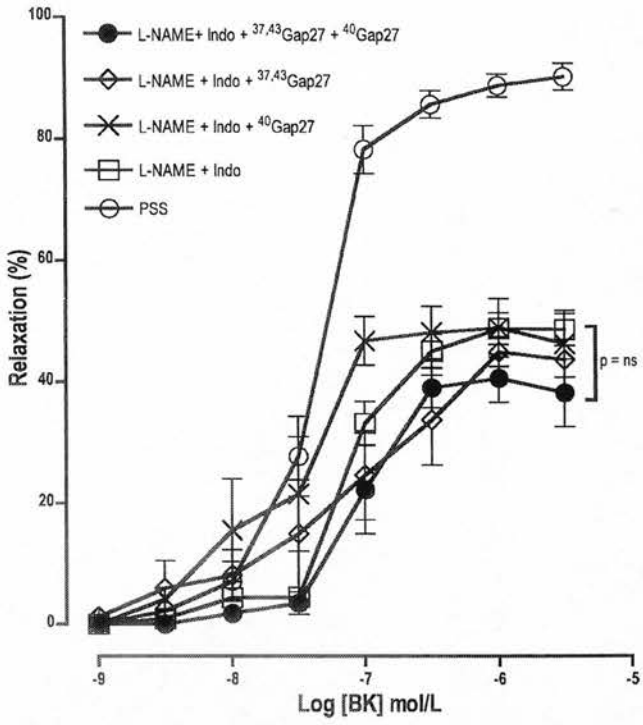

Fig. 2. Concentration-response curves for BK in subcutaneous arteries incubated in PSS (n = 18), L-NAME and indomethacin alone (n = 25), or L-NAME and indomethacin plus ^{37,43}Gap27 and ^{40}Gap27 combined (450 μmol/l each; n = 5), ^{37,43}Gap27 alone (900 μmol/l; n = 5), or ^{40}Gap27 alone (900 μmol/l; n = 4). P > 0.05, L-NAME and indomethacin alone vs. L-NAME and indomethacin in combination with ^{37,43}Gap27 and/or ^{40}Gap27; ns, not significant.

Effects of ^{43}Gap26 alone on endothelium-dependent vasorelaxation. Incubation with ^{43}Gap26 (900 μmol/l) (without L-NAME and indomethacin) reduced maximal BK-induced relaxation compared with the same arteries incubated in PSS alone (R_{max}: 40.6 ± 15.8% with ^{43}Gap26 alone vs. 85.8 ± 5.5% in PSS alone, P = 0.039, n = 5). Maximal BK-induced relaxation after incubation with ^{43}Gap26 (900 μmol/l) alone was not significantly different from that observed after incubation with the three combined CMPs (each at 300 μmol/l) in PSS (R_{max}: 40.6 ± 15.8% with ^{43}Gap26 alone vs. 46.6 ± 2.8% with combined CMPs in PSS, P = 0.69).

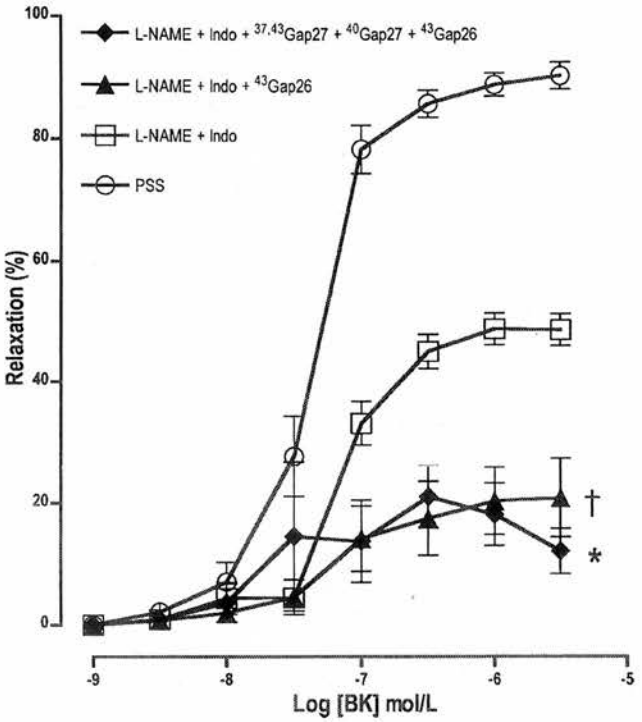

Fig. 3. Concentration-response curves for BK in subcutaneous arteries incubated in PSS (n = 18), L-NAME and indomethacin alone (n = 25), or L-NAME and indomethacin plus ^{37,43}Gap27, ^{40}Gap27, and ^{43}Gap26 combined (300 μmol/l each; n = 6) or ^{43}Gap26 alone (900 μmol/l; n = 5). *$P < 0.001$; †$P < 0.005$ vs. L-NAME and indomethacin alone.

Endothelium-independent vasorelaxation and vasoconstriction. Relaxation evoked by the exogenous NO donor SNP (0.1 mmol/l) was unaffected throughout all studies (data not shown). NE induced vasoconstriction of 2.1 ± 0.07 mN/mm^2 and was unaffected by any of the incubation protocols. ^{43}Gap26 alone had no effect on responses to the endothelium-independent vasodilators SNP and pinacidil (n = 5 for both) (Fig. 4).

Immunohistochemistry. Immunohistochemistry demonstrated the consistent presence of Cx37, Cx40, and Cx43 in arterial segments (Fig. 5). Connexins 37, 40, and 43 were seen in both vascular smooth muscle and endothelial cells, although Cx40 was predominantly found in the endothelium.

DISCUSSION

We have made the first assessment of the role of gap junctions and connexin subtypes in EDHF-mediated vasorelaxation of human arteries by using highly specific gap junction inhibitors. We have shown that EDHF-mediated vasorelaxation relies on gap junction communication acting principally through the Cx43 subtype.

We have confirmed that subcutaneous resistance arteries obtained from pregnant women retain ~50% of their endothelium-dependent relaxant capacity after the inhibition of endothelial NO and PGI_2 synthesis with L-NAME and indomethacin, respectively. This L-NAME- and indomethacin-insensitive component of endothelium-dependent vasorelaxation is widely regarded to reflect EDHF activity. Furthermore, incubation

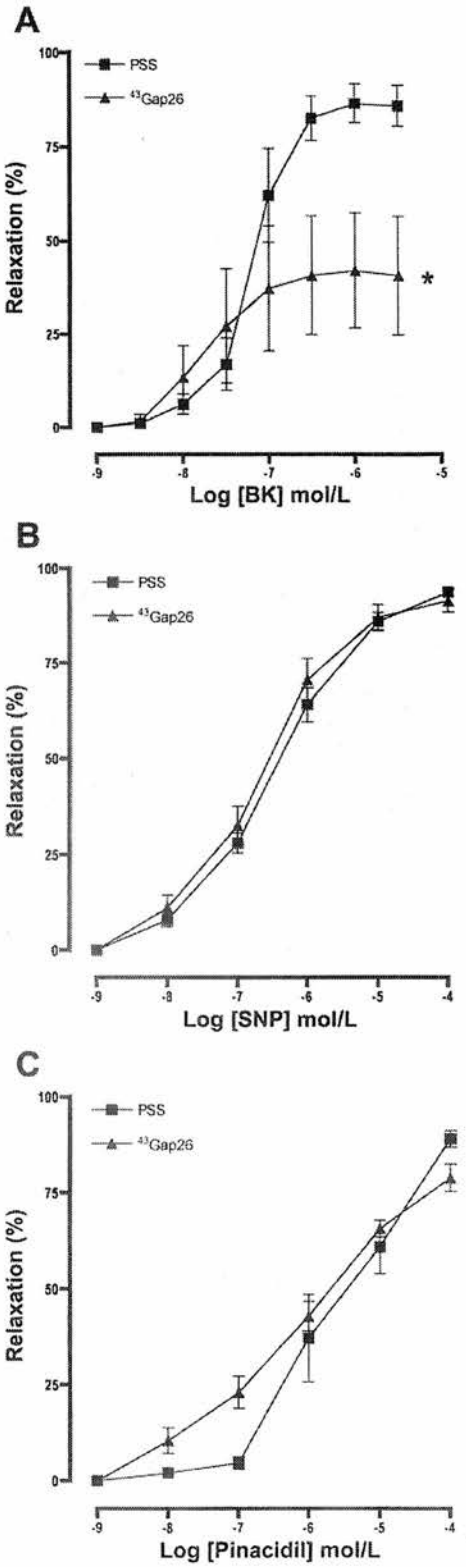

Fig. 4. Concentration-response curves for BK (*A*), sodium nitroprusside (SNP; *B*), and pinacidil (*C*) in subcutaneous arteries incubated in PSS and ^{43}Gap26 (900 μmol/l) (n = 5 for each). *$P < 0.05$ vs. PSS.

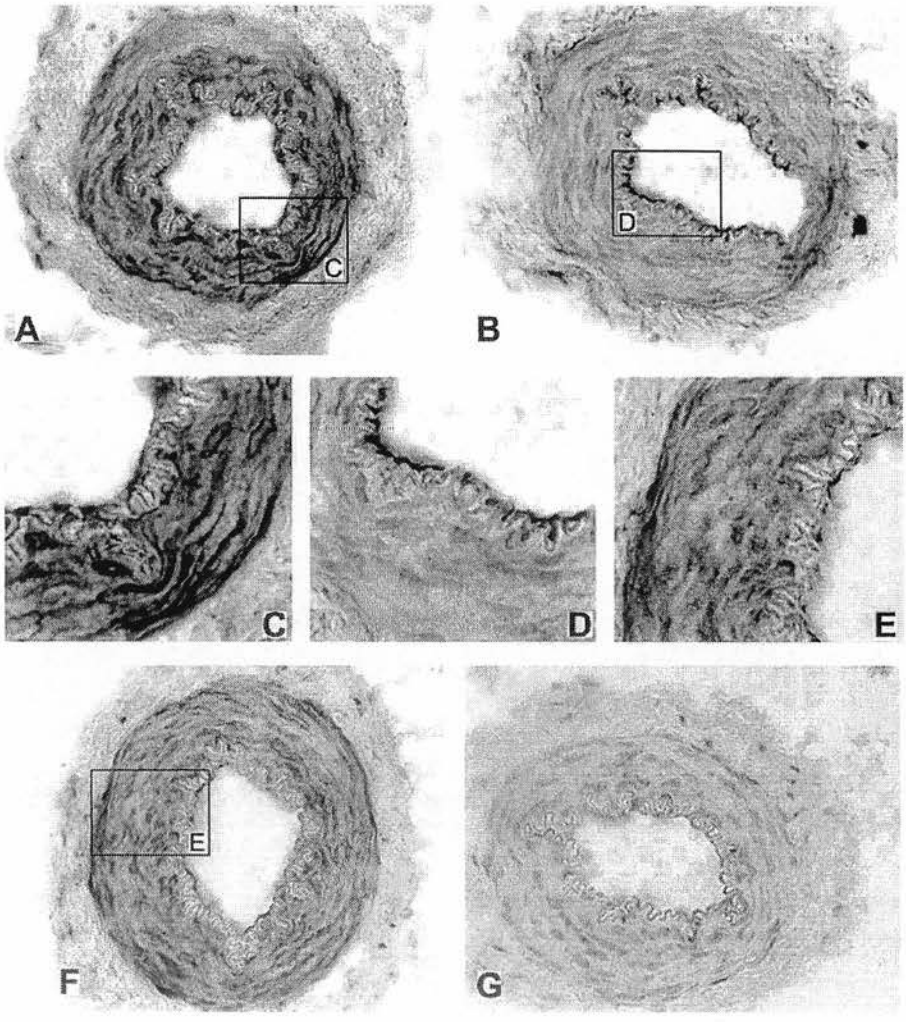

Fig. 5. Immunohistochemistry of pregnant human subcutaneous resistance arteries. *A* and *C*: staining for Cx43 shown in both smooth muscle and endothelial cell layers. *B* and *D*: staining for Cx40 principally shown in the endothelium. *E* and *F*: staining for Cx37 shown in endothelium and smooth muscle. *G*: control images of arteries incubated without primary antibodies showing no staining for connexins. All images, magnification ×40.

with a combination of ^{37,43}Gap27, ^{40}Gap27, and ^{43}Gap26 (300 μmol/l each) attenuated vasorelaxation to BK to a similar extent as L-NAME and indomethacin alone and revealed that gap junctions are required for ~50% of BK-induced vasorelaxation.

Coincubation of the triple CMP combination with L-NAME and indomethacin virtually abolished BK-induced vasorelaxation. That is, the L-NAME/indomethacin-insensitive component of BK-induced relaxation was inhibited by gap junction blockade. This provides further evidence of a pivotal role for gap junctions in EDHF-mediated relaxation in these vessels. These findings are in keeping with our group's previous study (17) in which BK-mediated relaxation was abolished by a combination of L-NAME, indomethacin, and 18α-glycyrrhetinic acid.

We went on to assess the effects of each of the three CMPs to infer the relative functional importance of Cx37, Cx40, and Cx43. ^{43}Gap26 exerted marked inhibition of EDHF-mediated relaxation to BK. Indeed, at 900 μmol/l, its inhibitory effect did not differ from that seen after incubation with the triple combination of CMPs. The marked inhibition of vasodilatation achieved by incubation with ^{43}Gap26 contrasts with the lack of inhibition seen after incubation with ^{37,43}Gap27 and ^{40}Gap27 in combination (450 μmol/l each) and individually (900 μmol/l). Furthermore, in the absence of L-NAME and indomethacin, ^{43}Gap26 caused a significant attenuation in maximal vasodilatation to BK. Together, these findings suggest that the combined inhibitory effect of all three CMPs is derived predominantly from ^{43}Gap26 activity and implicate Cx43 as the dominant connexin isoform involved in the mediation of EDHF responses in these vessels.

Data from combined endothelial NOS/COX knockout mice imply a greater role for EDHF in females than in males (29), whereas other animal models have demonstrated dynamic Cx43 expression under the influence of estrogen. Pregnant rats exhibit enhanced EDHF-mediated vasodilatation and upregulation of vascular Cx43 expression (8). These effects are markedly diminished in ovariectomized animals but can be restored by exogenous estrogen supplementation (16). Furthermore, an increase in Cx43 expression in response to increased shear stress and mechanical load has been demonstrated (26). The rise in circulating blood volume associated with normal human pregnancy could invoke similar changes in Cx43 expression and function and account for the predominant func-

tional importance of Cx43 in our study. Although diversity exists between species and the sexes, EDHF makes a large contribution to endothelium-dependent vasodilatation in various vascular beds in men and nonpregnant women (3, 7, 21, 24, 25, 30, 31). The present study supports a clear role for Cx43 in vascular responses of pregnant women (8). Whether Cx43 is of such fundamental importance in men and nonpregnant women and in other vascular beds remains to be established.

The interpretation of results using older putative gap junction blockers has been limited by their nonspecific effects. Indeed, 18α-glycyrrhetinic acid can affect ion channel conduction to attenuate endothelial cell hyperpolarization directly. Confidence in the CMPs is growing: they do not suppress endothelial hyperpolarization directly and do not exert non-gap-junctional effects (12, 19). Their exact mechanism of action remains unclear, but they are thought to interfere with connexin-gating properties. Unlike 18α-glycyrrhetinic acid, they do not affect the structural integrity, number, or distribution of connexins (1). When administered in combination and individually in this study, the CMPs did not have any influence on vasorelaxation to SNP or preconstriction to NE, suggesting that they specifically inhibit endothelium-dependent vasorelaxation without interfering with vascular smooth muscle function. Whereas ^{43}Gap26 alone significantly attenuates maximal relaxation to BK, it did not have any influence on the concentration-response curve obtained after exposure to the endothelium-independent vasodilators SNP and pinacidil. This provides more robust evidence that this CMP does not exert nonspecific or toxic effects on smooth muscle function.

If Cx43 has a dominant role, then one might expect EDHF-mediated vasorelaxation to be inhibited by ^{37,43}Gap27 as well as ^{43}Gap26. ^{37,43}Gap27 shares sequence homology with the Gap27 domain of both Cx37 and Cx43, whereas ^{43}Gap26 shares homology with the Gap26 domain of Cx43 only. Both ^{37,43}Gap27 and ^{43}Gap26 have been shown to act in a connexin-specific manner (1, 5), but our findings concur with others who have reported differential activity between these two CMPs. In 2003, Chaytor et al. (4) reported that ^{37,43}Gap27 and ^{40}Gap27 partially inhibited the transmission of hyperpolarization from endothelium to smooth muscle in the rabbit iliac artery. However, the addition of ^{43}Gap26 was required to abolish this phenomenon completely, implying that ^{37,43}Gap27 causes incomplete inhibition of Cx43 (4). Because each of these peptides shares homology with different extracellular loops, these data suggest that targeting ^{37,43}Gap27 and ^{43}Gap26 vary in their propensity to inhibit Cx43 by acting at different sites. It is unclear whether Gap26 plays a greater role than Gap27 in Cx43 gating and/or docking.

Immunohistochemistry revealed Cx37 and Cx43 expression in both the endothelium and smooth muscle. Cx40 was also seen in these cells, but expression was predominantly seen in the endothelium. Although myoendothelial gap junctions are proposed to allow the transfer of hyperpolarization generated in the endothelium to the underlying smooth muscle, homocellular gap junctions between adjacent smooth muscle or endothelial cells are also an ideal conduit for the longitudinal conduction of hyperpolarization and coordinated regulation of vascular tone. Indeed, mice genetically engineered to lack vascular Cx40 display not only hypertension but also irregular vasomotion (9). Our own study has not differentiated whether the inhibition of EDHF-mediated vasodilatation is due to the inhibition of Cx43 in myoendothelial junctions, in homocellular junctions, or in both. Irrespective, we have demonstrated a critical role for this connexin in endothelium-dependent vasorelaxation. Furthermore, we have shown that endothelium-independent vasorelaxation is unaffected by gap junction and, specifically, Cx43 inhibition.

In summary, we have shown that EDHF accounts for ~50% of BK-induced vasorelaxation in resistance arteries from pregnant women. Using CMPs, we have provided functional evidence that EDHF mediates its vasorelaxatory actions via gap junctions. We have demonstrated the expression of three major connexin isoforms and highlighted a predominant functional role for Cx43 in the mediation of EDHF responses in this vascular bed. The extension of these studies to men, nonpregnant women, and pathophysiological states warrants further investigation.

GRANTS

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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 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 receptor agonist, 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 dilation, venous constriction, platelet activation, and tissue-type plasminogen activator (t-PA) release (9). 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.

Methods

Subjects. A total of 30 healthy nonsmokers (mean age 22 years; 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 volunteers. Participants were screened and excluded for clinically significant conditions including hypertension, hyperlipidemia, 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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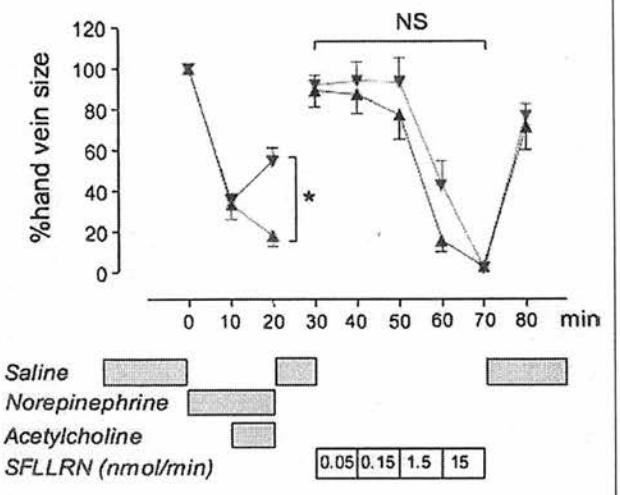

Figure 1 Dorsal Hand Vein Responses to Acetylcholine and SFLLRN (After Pre-Constriction With Norepinephrine)

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 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 (Fig. 1) ($p = 0.09$; ANOVA).

Endothelium-derived vasodilators and PAR-1–induced vasodilation. SFLLRN caused an increase in forearm blood flow that was unaffected by PGI_2 inhibition with oral aspirin (600 mg) (Fig. 2A). The NO synthase inhibitor, L-NMMA, caused ~50% decrease in basal forearm blood flow (from 3.04 ± 0.37 ml/100ml tissue/min to 1.49 ± 0.19 ml/100ml tissue/min; $p < 0.001$). Intrabrachial SNP (90 to 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 SNP versus baseline; paired Student *t* test). The inhibition of endogenous NO synthesis by the NO clamp attenuated SFLLRN-induced vasodilation (Fig. 2B).

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

Both with and without concurrent NO synthase and PGI_2 synthase inhibition, TEA attenuated, but did not abolish arterial vasodilation to the control endothelium-dependent vasodilator, bradykinin (Figs. 3A and 3B). Tetraethylammonium ion did not affect endothelium-independent forearm arterial 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

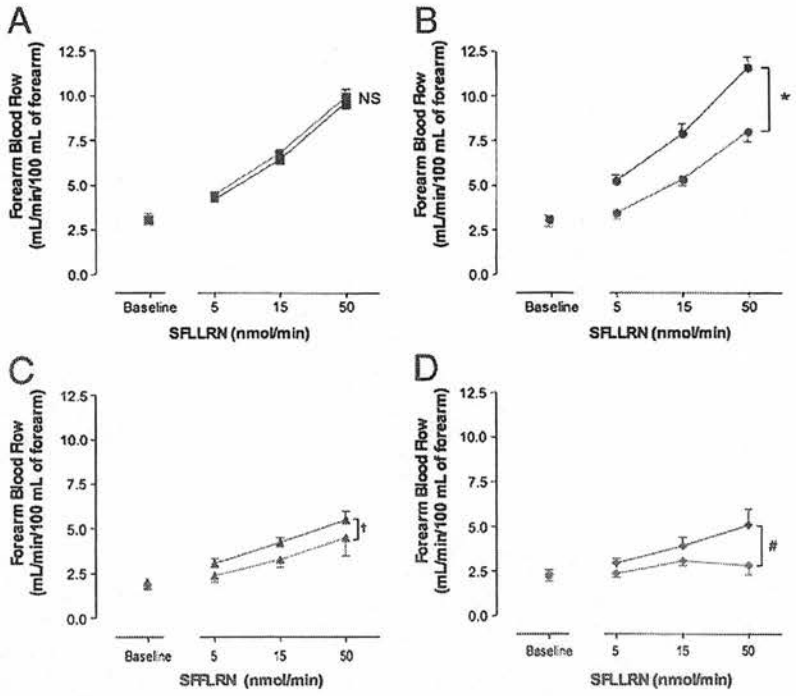

Figure 2 SFLLRN-Induced Forearm Arterial Vasodilation

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)**, †$p < 0.05$ 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).

endothelium, 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 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 3 main known mediators of endothelium-dependent vasodilation: PGI_2, NO, and EDHF. Although PGI_2 inhibition appeared to have no effect, inhibition of NO and potassium channels both attenuated the PAR-1–induced vasodilation. 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 the PAR-1 agonist. 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. 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 (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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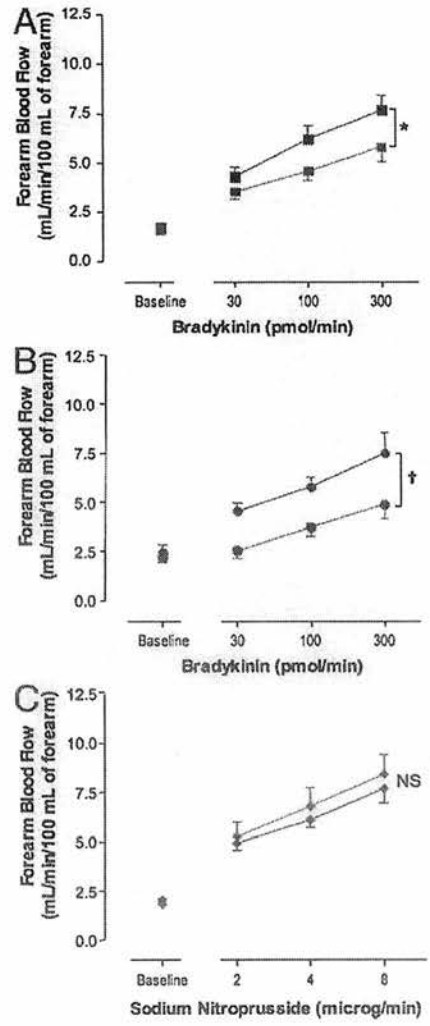

Figure 3 Bradykinin- and SNP-Induced Forearm Arterial Vasodilation

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.

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 (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

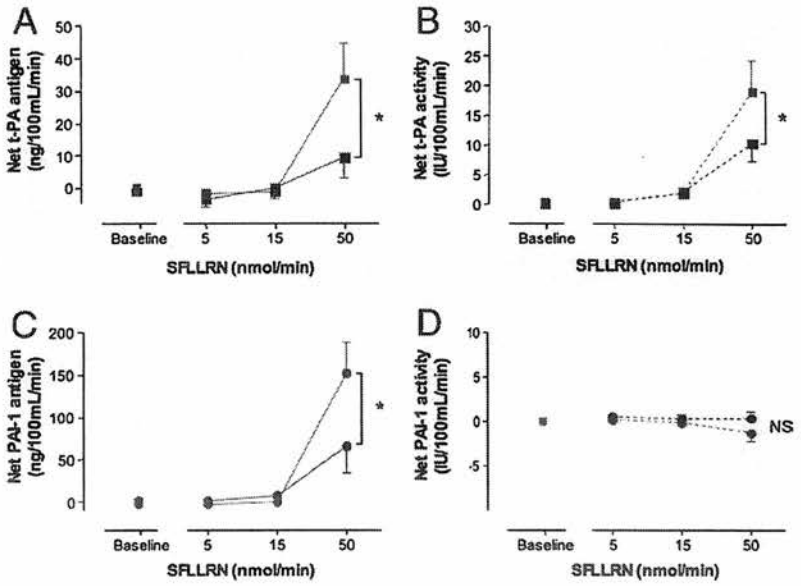

Figure 4 SFLLRN-Induced t-PA and PAI-1 Release

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.

previously shown that SLIGKV, a highly selective PAR-2 activating peptide, causes only modest arterial vasodilation at high doses and, in contrast to PAR-1 activation, causes marked venodilation and does not cause arterial t-PA release in vivo (24). 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 median effective dose for the PAR-2 receptor (23). However, we do accept that, in future studies, consideration should be given to the use of the more selective PAR-1 activating peptide, TFLLRN (23,25).

The role of NO in bradykinin-induced t-PA release remains controversial (19) and the present study has not definitively addressed this issue. Although NO donors do not induce t-PA release (26,27), inhibition of NO synthesis has been reported either to have no effect (21), or to increase (20), bradykinin-induced t-PA release. Our own unpaired analysis of data from different subject populations is in keeping 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.

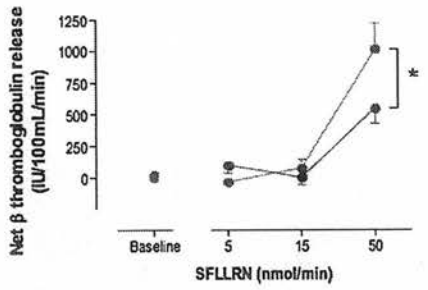

Figure 5 SFLLRN-Induced Beta-Thromboglobulin Release

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.

Acknowledgments

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The vascular effects of rotigaptide *in vivo* in man

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ABSTRACT

Endothelium-derived hyperpolarising factor (EDHF) causes vasorelaxation and may contribute to the release of the endogenous fibrinolytic factor, tissue-plasminogen activator (t-PA). Rotigaptide enhances communication via the connexin 43 gap junction subunit and may potentiate the vascular actions of EDHF. The aims of the present study were therefore to determine whether rotigaptide influences basal and stimulated endothelium-dependent vasodilatation and t-PA release *in vivo* in man.

Using venous occlusion plethysmography, forearm blood flow was measured in 27 healthy volunteers during intra-brachial infusions of rotigaptide (0.25–25 nmol/min) alone, or co-administered with endothelium-dependent (acetylcholine [5–20 μg/min] and bradykinin [30–300 pmol/min]) and independent (sodium nitroprusside [2–8 μg/min]) vasodilators in the presence or absence of aspirin and the 'nitric oxide clamp'. The 'nitric oxide clamp' inhibits endogenous nitric oxide synthesis with L-N-monomethylarginine and restores resting blood flow with the exogenous nitric oxide donor, sodium nitroprusside.

Basal blood flow was unaffected by rotigaptide (P = NS). Acetylcholine, bradykinin and sodium nitroprusside all caused dose-dependent vasodilatation in the presence and absence of aspirin and the 'nitric oxide clamp' ($P \leq 0.005$ for all). These responses were unaffected by rotigaptide (P = NS). Bradykinin caused t-PA antigen and activity release ($P = 0.04$, $P < 0.0001$, respectively) that was unaffected by rotigaptide.

Augmentation of connexin 43 communication has no effect on basal vascular tone and does not enhance endothelium-dependent or independent vasodilatation, or t-PA release in the forearm arterial circulation of healthy men. It remains to be established whether augmentation of connexin 43 communication improves endothelial function in patients with vascular disease.

1. Introduction

The endothelium plays a major role in the regulation of vascular tone and is responsible for the release of the endogenous fibrinolytic factor, tissue-plasminogen activator (t-PA). The elucidation of their roles in vascular physiology and pathophysiology has been fundamental to recent advances in the treatment and prevention of many cardiovascular diseases.

After blockade of both nitric oxide and prostacyclin generation, a substantial degree of endothelium-dependent vasodilatation remains and is attributed to endothelium-derived

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hyperpolarising factor (EDHF) [1]. EDHF activity has been demonstrated in the microcirculation of a variety of human vascular beds including the peripheral [2], mesenteric [3] and subcutaneous [4,5] circulations in addition to the coronary [6,7] and renal [8] vasculature. Consistently, its contribution is most prominent in the small resistance arteries [3,9] that regulate systemic blood pressure and local tissue perfusion. Furthermore, the release of t-PA from the endothelium is independent of both nitric oxide and prostacyclin production [10,11], and it has been suggested that EDHF may mediate its release [11,12]. Thus, alterations in EDHF activity may contribute to endothelial dysfunction and its manipulation presents an exciting opportunity to restore vascular health and reduce the burden of cardiovascular disease [13].

The transmission of hyperpolarisation from the endothelium to the underlying smooth muscle occurs via undefined mediators and pathways. Various mediators have been proposed but none has emerged as a universal EDHF in all species and vascular beds [14,15]. However, there is considerable evidence that gap junctions are required [15–20]. These aqueous pores are found at points of cell–cell contact and allow the intercellular transfer of small molecules (<1 kDa). Myoendothelial gap junctions are, therefore, ideally suited to the radial transfer of electronic charge or second messenger between the endothelium and underlying smooth muscle. Furthermore, gap junctions are particularly abundant in the microcirculation where the density of their expression correlates with an increasing contribution of EDHF to endothelial vasodilatation in these small resistance vessels [21]. Each gap junction hemichannel is composed of six connexin subunits including connexin (Cx) 37, 40 and 43 in the mammalian vasculature. Indeed, using specific connexin antagonist peptides, we have previously demonstrated the critical importance of Cx43 in EDHF-mediated vasodilatation of human resistance arteries *in vitro* [5].

It has not previously been possible to make a direct assessment of the role of gap junctions in the mediation of EDHF responses *in vivo* in man. However, rotigaptide (ZP-123) is a synthetic hexapeptide (Ac-D-Tyr-D-Pro-D-Hyp-Gly-D-Ala-Gly-NH_2) that alters the phosphorylation status of Cx43 to potentiate communication via gap junctions [22–25]. In addition to its clinical development as an anti-arrhythmic agent [26,27], it has been promoted as an important new tool to aid in the dissection of the physiologic role of gap junctions [22]. Here, we have conducted the first clinical assessment of the role of gap junctions, and specifically Cx43, in the peripheral vascular EDHF-mediated responses. We tested the hypothesis that, by increasing communication via gap junctions, rotigaptide would enhance EDHF-mediated vasodilatation and t-PA release in the forearm arterial circulation of healthy man.

2. Methods

2.1. *Preliminary validation study*

The biological activity of rotigaptide was assessed through *in vitro* measurements of its effect on transmural conduction velocity in rabbit ventricular myocardium. Hearts from four male New Zealand White rabbits were used for these experiments, which conform to the standards set out in the Animals (Scientific Procedures) Act 1986.

Rabbits ($n = 4$) were killed with a single intravenous injection of 100 mg/kg pentobarbital sodium (Rhône Mérieux, France). Hearts were excised, placed in chilled Tyrode's solution (containing [mmol/L]: Na 134.5, Mg 1.0, K 5.0, Ca 1.9, Cl 101.8, SO_4 1.0, H_2PO_4 0.7, HCO_3 20, acetate 20 and glucose 10) and perfused via the left coronary artery with oxygenated (95% O_2–5% CO_2) Tyrode's solution maintained at pH 7.4 and 37 °C. Perfused left ventricular free wall wedge preparations were dissected out and mounted in a custom built chamber which allowed access to the transmural surface for imaging. The preparation was stimulated using a bipolar electrode placed on the epicardial surface at 1.5× diastolic threshold using a 2 ms pulse at a basic cycle length of 350 ms. Perfusion pressure and electrocardiogram were monitored throughout. An optical mapping system was used to record optical action potentials as previously described [28]. Motion artifact was minimised using 15 mmol/L 2,3 butanedione monoxime (BDM; Sigma Aldrich, UK). Measurements were taken at 15-min intervals. After two control recordings the perfusate was changed to Tyrode's solution containing BDM and 1 μmol/L rotigaptide for a further 15 min and measurements were repeated.

2.2. *Clinical study*

This clinical study was performed with the approval of the local research ethics committee in accordance with the Declaration of Helsinki and with the written informed consent of each subject.

2.2.1. *Subjects*

Healthy non-smokers (mean age 22 years; range 19–25 years) were recruited into the study. Participants were excluded if they had clinically significant conditions including hypertension, hyperlipidemia, 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. On the day of study, participants had fasted and abstained from caffeine and tobacco for at least 4 h and from alcohol for 24 h.

2.2.2. *Drugs*

Pharmaceutical grade bradykinin (Clinalfa AG, Läufelfingen, Switzerland), acetylcholine (Novartis Ltd., Middlesex, UK), L-N(G)-monomethyl arginine citrate (Clinalfa), sodium nitroprusside (Hospira Inc., CA, USA) and rotigaptide (American Peptide Inc., CA, USA) were dissolved in physiological saline. Aspirin was obtained from Dagra Pharma, Diemen, Netherlands.

2.2.3. *Forearm venous occlusion plethysmography*

All subjects underwent cannulation of the brachial artery with a 27G-standard wire steel needle under controlled conditions. All studies were performed with patients lying supine in a quiet, temperature controlled (22–25 °C) room. 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 [29,30]. Supine heart rate and blood pressure were monitored at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer.

2.2.4. *Intra-arterial drug administration*

2.2.4.1. *PROTOCOL 1. Effect of rotigaptide on basal forearm blood flow.* Five participants attended once each. They received intra-arterial rotigaptide at 0.25, 0.75, 2.5, 7.5 and 25 nmol/min for 6 min at each dose. Assuming basal forearm blood flow of 25 mL/min, this would give tissue rotigaptide concentrations of 0.01–1.0 μmol/L. Forearm blood flow was measured during the final 3 min of infusion of each dose.

2.2.4.2. *PROTOCOL 2. Effect of rotigaptide on agonist-induced vasodilatation and tissue-plasminogen activator release.* Twelve volunteers attended on each of three occasions. After a 20-min intra-arterial infusion of 0.9% saline, participants received either intra-arterial placebo (0.9% saline), 2.5 nmol/min rotigaptide or 25 nmol/min rotigaptide using a randomised double-blind cross-over design. Rotigaptide or placebo was administered alone for 20 min before being co-infused with ascending doses of bradykinin (an endothelium-dependent vasodilator that causes the release of tissue-plasminogen activator; 30–300 pmol/min), acetylcholine (an endothelium-dependent vasodilator that does not cause the release of t-PA; 5–20 μg/min) and sodium nitroprusside (an endothelium-independent vasodilator that does not cause the release of t-PA; 2–8 μg/min). Co-infused drugs were separated by a 20-min infusion of 0.9% saline. The order of agonist infusion was randomised between participants but maintained constant for each of the three visits.

2.2.4.3. *PROTOCOL 3. Effect of rotigaptide on EDHF-mediated vasodilatation.* Ten volunteers were recruited to attend on two occasions. EDHF activity was isolated by inhibiting the production of both prostacyclin and nitric oxide on each of the two visits. Cyclo-oxygenase activity was inhibited with a single 600 mg dose of oral aspirin 1 h prior to each study. Nitric oxide production was inhibited with L-N(G)-monomethyl arginine citrate (L-NMMA) in the 'nitric oxide clamp'. After a 20-min intra-arterial infusion of 0.9% saline, L-NMMA (8 μmol/min) was infused via the brachial artery. To compensate for L-NMMA induced basal vasoconstriction, forearm blood flow was restored to baseline using a titrated dose of exogenous nitric oxide in the form of intra-brachial sodium nitroprusside (SNP; 90–900 ng/min). The titrated dose of SNP was co-infused with L-NMMA throughout the study. This arrangement allows a constant 'clamped' delivery of exogenous nitric oxide whilst endogenous nitric oxide synthase activity is inhibited [31].

Either rotigaptide (25 nmol/min) or saline placebo was co-infused with the 'nitric oxide clamp' in a double-blind randomised cross-over design. Subsequently, ascending doses of bradykinin (30–300 pmol/min), acetylcholine (5–20 μg/min) and sodium nitroprusside (2–8 μg/min) were co-infused and separated by a 20-min infusion of 0.9% saline. The order of agonist co-infusion was randomised between participants but maintained constant between visits.

2.2.5. *Blood sampling*

Seventeen-gauge venous cannulae were inserted into left and right ante-cubital fossae during Protocol 2. Blood samples were drawn simultaneously from each arm before bradykinin infusion and after the maximum dose of bradykinin (300 pmol/min). Blood was collected into acidified buffered citrate (Stabilyte, Biopool International, UK; for t-PA assays) and into citrate (BD Vacutainer, BD UK Ltd., UK; for measurement of t-PA's major endogenous inhibitor, plasminogen activator inhibitor type 1 [PAI-1]). Samples were kept on ice before centrifugation at 2000 × *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, 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. Hematocrit was measured at baseline and at the end of the study.

2.3. *Data analysis and statistics*

2.3.1. *Optical mapping validation study*

Data analysis was performed using custom software. Activation time was determined at the midpoint between baseline and the peak of the action potential upstroke. Transmural conduction velocity was calculated for each time point using activation time from the earliest activation on the epicardial edge of the transmural surface to the earliest activation on the endocardial side.

2.3.2. *Clinical study*

Forearm plethysmographic data were analyzed as described previously [29]. Estimated net release of plasma t-PA and PAI-1 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 non-infused arms [32].

Variables are reported as mean ± S.E.M. and analyzed 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.

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

3. Results

3.1. *Effect of rotigaptide on myocardial conduction velocity in vitro*

In all experiments, transmural conduction velocity remained constant during control perfusion (20.8 ± 1.7 cm/s versus 21.4 ± 1.6 cm/s, P = NS). Following perfusion with rotigaptide, an increase in transmural conduction velocity was observed in rabbit ventricular tissue *in vitro* (29.4 ± 1.7 cm/s, $P < 0.001$, ANOVA; Fig. 1).

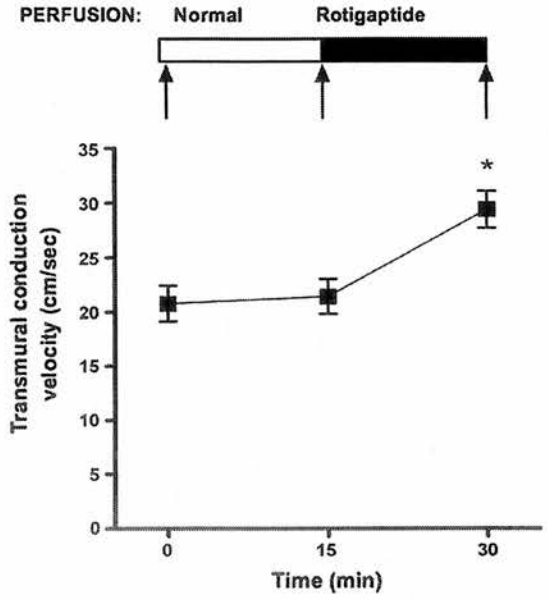

Fig. 1 – Effect of 1 μmol/L rotigaptide on transmural conduction velocity in perfused rabbit ventricular myocardium. *P < 0.001, 30 min versus 0 or 15 min, t-test.

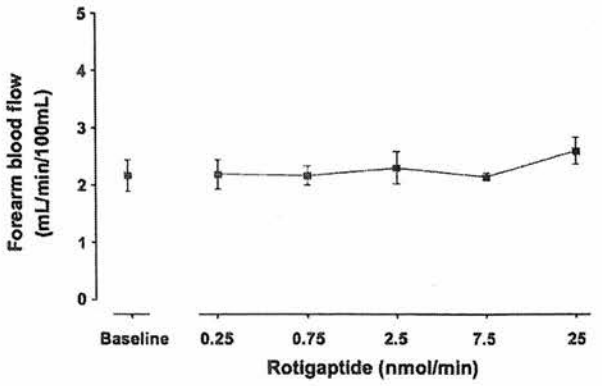

Fig. 2 – Forearm blood flow during intra-brachial infusion of rotigaptide (0.25–25 nmol/min).

3.2. *Effect of rotigaptide on basal forearm blood flow*

At doses of 0.25–25 nmol/min, rotigaptide had no effect upon basal forearm blood flow (P = NS, ANOVA: Fig. 2).

3.3. *Effect of rotigaptide on agonist-induced vasodilatation and tissue-plasminogen activator release*

Acetylcholine, bradykinin and sodium nitroprusside each caused dose-dependent arterial vasodilatation ($P < 0.0001$ for all, ANOVA) that was unaffected by either 2.5 or 25 nmol/min rotigaptide (P = NS for both, in the presence versus the absence of rotigaptide; ANOVA: Fig. 3).

At baseline, net t-PA antigen release was 0.09 ± 0.05 ng/100mL/min and net release of t-PA activity was 0.54 ± 1.4 IU/100 mL/min. This was unchanged by the presence of rotigaptide (P = NS for all; ANOVA). Bradykinin (300 pmol/min) caused the release of t-PA antigen (7.62 ± 5.56 ng/100 mL/min; P = 0.04, ANOVA) and activity (6.15 ± 2.20 IU/100 mL/min; $P < 0.0001$, ANOVA) but this was unaffected by either 2.5 or 25 nmol/min rotigaptide (P = NS for all, ANOVA).

Net release of PAI-1 antigen and activity was unaffected by bradykinin (300 pmol/min) in the presence or absence of rotigaptide (P = NS for all, ANOVA; data not shown).

3.4. *Effect of rotigaptide on EDHF-mediated vasodilatation*

Intra-arterial L-NMMA (8 μmol/min) reduced basal forearm blood flow by approximately 38% (from 2.40 ± 0.20 reduced to 1.50 ± 0.20 mL/100 mL tissue/min; $P < 0.0001$) and was unaffected by rotigaptide (P = NS, 2-way ANOVA). Forearm blood flow was restored to baseline levels by the titration of sodium nitroprusside (P = NS, ANOVA).

After the inhibition of endothelial nitric oxide and prostacyclin synthesis, dose-dependent vasodilatation was evoked by acetylcholine, bradykinin and sodium nitroprusside ($P \leq 0.005$ for all, ANOVA). This response was not altered by the presence of rotigaptide (P = NS, ANOVA; Fig. 4).

4. Discussion

For the first time, we have assessed the role of connexin 43 and gap junctions in the control of vascular tone and t-PA release *in vivo* in man. We have shown, in healthy volunteers, that potentiation of connexin 43-mediated intercellular communication with rotigaptide does not affect basal forearm arterial

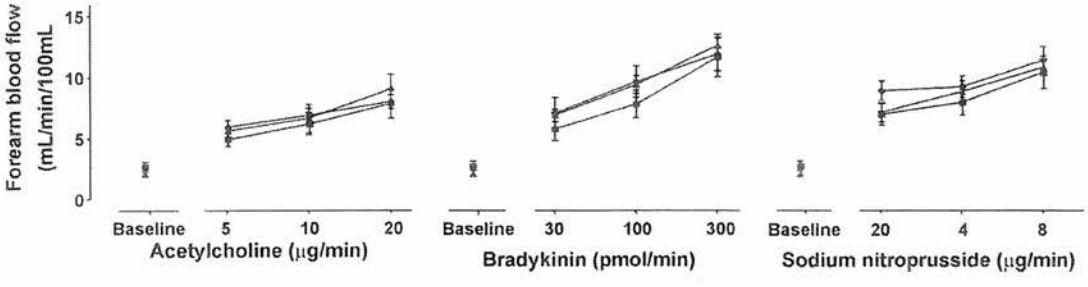

Fig. 3 – Forearm blood flow during intra-brachial infusion of acetylcholine (left hand panel), bradykinin (middle panel) and sodium nitroprusside (right hand panel) in the presence of placebo (squares), 2.5 nmol/min rotigaptide (triangles) or 25 nmol/min rotigaptide (circles). P = NS for rotigaptide versus placebo, ANOVA.

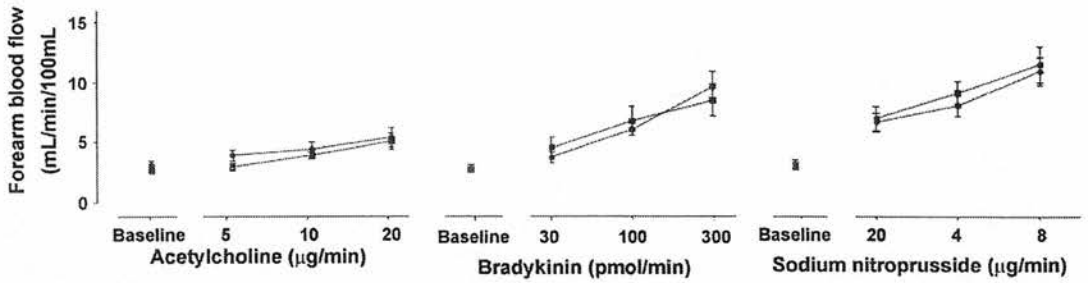

Fig. 4 – In the presence of the 'nitric oxide clamp' and oral aspirin, forearm blood flow during intra-brachial infusion of acetylcholine (left hand panel), bradykinin (middle panel) and sodium nitroprusside (right hand panel) in the presence of placebo (squares) or 25 nmol/min rotigaptide (circles). P = NS for rotigaptide versus placebo, ANOVA.

blood flow or agonist-induced vasodilatation and t-PA release in the presence or absence of concomitant inhibition of prostacyclin and nitric oxide production.

To date, there has been no direct assessment of the role of gap junctions, nor individual connexin subtypes, in the regulation of vascular function *in vivo* in man. The use of connexin antagonists has been limited by concerns about the potential for toxicity and older putative gap junction blockers exert a range of non-endothelial effects and alter ion channel permeability [33,34]. However, rotigaptide offers a novel means for the assessment of gap junction-dependent phenomena *in vivo*. Not only has it been safely administered to healthy humans [26,27] but its actions are specific to Cx43. Indeed, it augments communication via gap junctions in the absence of changes in membrane conduction or basal current [35], and it exhibits no binding to a large array of receptors including numerous ion channels [36]. It enhances intercellular dye transfer between HeLa cells expressing Cx43 but not between those expressing Cx26 or Cx32 [23] and promotes electrical coupling between ventricular myocytes via alterations in the phosphorylation status of Cx43 [25,37]. Indeed, phopshorylation of Cx43 maintains it an open state [38] and Kjølbye et al. have recently demonstrated that the anti-arrhythmic effects of rotigaptide are associated with the inhibition of Cx43 dephosphorylation [25].

We have previously demonstrated that EDHF-mediated vasodilatation of subcutaneous resistance arteries from pregnant women depends upon the presence of functional Cx43 whilst Cx37 and Cx40 are not required [5]. Indeed, in comparison to Cx37 and Cx40, the vascular expression of Cx43 appears to be particularly labile. It is up-regulated by oestrogen [39], at the leading edge of atherosclerotic plaques [40], after vascular injury [41] and at sites of increased shear stress [42,43]. Furthermore, type I diabetes mellitus is associated with an impairment of EDHF-mediated vascular responses [13,44], and elevated glucose concentrations cause the isolated down-regulation of Cx43 expression [45] and permeability [46] *in vitro*. The examination of Cx43-mediated responses is, therefore, of particular relevance not only to our understanding of vascular physiology, but might also represent a major therapeutic target to attenuate endothelial dysfunction and improve vascular health.

Despite theoretical indications, we have failed to demonstrate a major vascular effect of rotigaptide even during inhibition of both nitric oxide and prostacyclin production. Was the rotigaptide inactive or used at an inadequate dose? Our preliminary *in vitro* electrophysiological optical mapping study provided important information confirming the biological activity of our preparation of rotigaptide at 1 μmol/L. The maximum dose of rotigaptide assessed in the clinical study was 25 nmol/min which, based upon forearm blood flow of 25 mL/min, equates to an estimated tissue concentration of approximately 1 μmol/L. This concentration is in excess of the effective anti-arrhythmic concentration employed in previous animal models [24,47–49] and is similar to the maximum tissue concentration of rotigaptide achieved in clinical trials [27]. Furthermore, it is in excess of the concentration recently shown to be required for the prevention of Cx43 dephosphorylation [25]. We therefore do not believe that the preparation was inactive or used at the wrong dose.

Connexin 43 is expressed abundantly in a wide range of resistance arteries obtained from a variety of species [15] including humans [5]. Whilst our previous assessment of the role of connexin subtypes in human EDHF-mediated responses demonstrated a critical role for connexin 43 and not connexin 37 or 40 [5], it is possible that, in the non-pregnant state, the vasomotor and EDHF mechanism requires a contribution from all of these subtypes. Rotigaptide specifically augments connexin 43-mediated signalling but the augmentation of vascular gap junction mediated communication may therefore require the combined potentiation of all three of the major vascular connexin subtypes.

The present study made an examination of vascular responses in healthy young men. It is conceivable that vasomotor and endogenous fibrinolytic responses cannot be augmented because the endothelium is already maximally active with a high baseline open-state probability for connexin 43. Indeed, the anti-arrhythmic activity of rotigaptide is particularly potent in the context of acidosis and metabolic stress during which the open-state probability of Cx43 is relatively low [36]. Furthermore, we were careful to dissect out EDHF-mediated activity by the inhibition of prostacyclin and nitric oxide with oral aspirin and the nitric oxide clamp respectively. EDHF has activity that is reciprocal to nitric oxide [50] and becomes up-regulated to compensate for impaired nitric oxide bioavailability in a variety of disease states [13]. Therefore, the argument that EDHF is maximally active in the studied population becomes more pertinent in the presence of nitric oxide inhibition.

The EDHF mechanism may differ between vascular beds but the forearm arterial model employed here has been predictive of the behaviour of the coronary vasculature in prior studies of other aspects of endothelial function [30,51,52]. However, whilst the present study provides important data regarding rotigaptide in the peripheral resistance vasculature, caution should still be applied when considering extrapolation of the results to infer similar pharmacodynamic effects in other vascular beds.

In conclusion, we have demonstrated that intra-arterial rotigaptide does not augment vasomotion or endogenous fibrinolysis in healthy subjects. Whether enhancement of connexin 43-mediated intercellular communication influences vascular function in conditions associated with specific impairment of EDHF-mediated activity, such as type I diabetes mellitus, remains to be evaluated. Not only would this assessment provide important mechanistic insights to the pathophysiology of endothelial dysfunction but could also highlight an important novel therapeutic target.

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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).

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

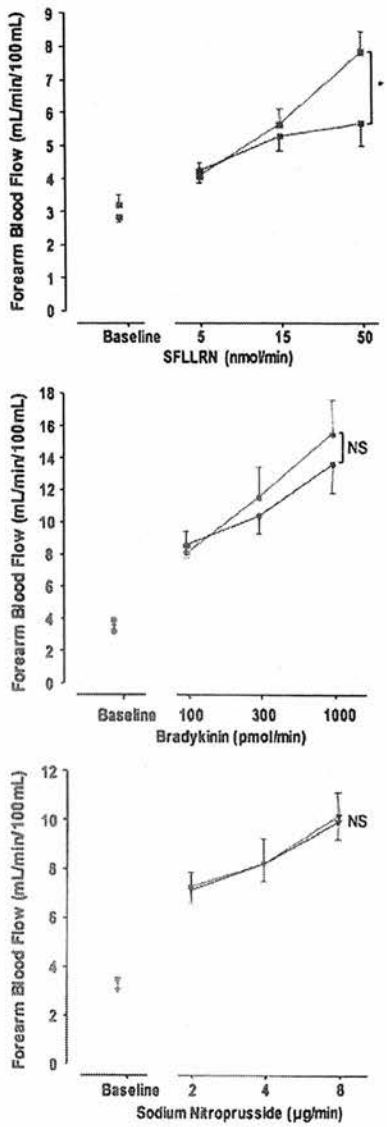

Figure 1 Forearm Arterial Vasodilation in Smokers and NonSmokers

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).

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 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.

tective 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.

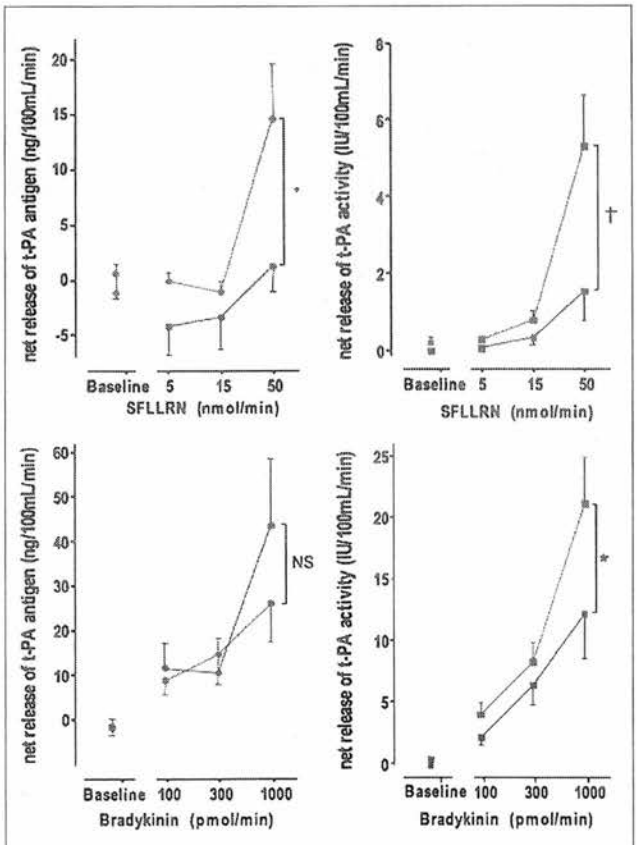

Figure 2 Net Release of t-PA Activator in Smokers and Nonsmokers

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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