UNIVERSITY OF EDINBURGH

STUDIES INTO ENDOGENOUS FIBRINOLYSIS IN THE PERIPHERAL AND CORONARY VASCULAR BEDS OF MAN

Dr David Ernest Newby, BA BSc (Hons) BM DM MRCP

Doctorate of Philosophy

Clinical Pharmacology Unit and Research Centre
University of Edinburgh
Western General Hospital
Edinburgh
EH4 2XU

Department of Cardiology
University of Edinburgh
Royal Infirmary
Edinburgh
EH3 9YW

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ABSTRACT

Background Acute myocardial infarction is caused by thrombotic occlusion of a coronary artery compromised by atheromatous plaque. The interplay between acute plaque rupture or erosion and the local haemostatic and fibrinolytic activities are critical determinants in the initiation and resolution of the thrombotic complications of coronary atheroma. This is exemplified by the high rate of spontaneous reperfusion in the infarct related artery after acute myocardial infarction.

Objectives The aims of the thesis were: first, to establish a selective, specific and reproducible model for assessing endothelial function and acute endogenous fibrinolytic capacity *in vivo* in the peripheral circulation of man; second, to characterise the underlying mechanisms of the fibrinolytic response in this model; third, to compare the acute endogenous fibrinolytic capacity in health and disease; fourth, to examine the influence of therapeutic intervention on the fibrinolytic response; and finally, fifth, to apply this model to the coronary circulation in order to determine the acute coronary fibrinolytic response and assess its relationship with the extent of coronary atheroma.

Methods *Peripheral circulation.* Blood flow and plasma fibrinolytic parameters were determined in both forearms using venous occlusion plethysmography and blood samples withdrawn from the antecubital fossae. The brachial artery of the non-dominant forearm was cannulated and intra-arterial drugs administered. Plasma fibrinolytic parameters were determined using enzyme linked immunosorbant and photometric methods. The mechanisms of substance P action were explored using neurokinin type 1 receptor antagonism, nitric oxide synthase inhibition and the nitric oxide donor, sodium nitroprusside. The acute fibrinolytic response was examined in habitual cigarette smokers and patients with hypercholesterolaemia: the latter were also assessed following 6 weeks of lipid lowering therapy. *Coronary circulation.* Following diagnostic coronary angiography, the proximal coronary artery plaque volume was determined using computerised three dimensional reconstruction of intravascular ultrasound images. Blood flow and fibrinolytic responses to selective left anterior descending coronary artery infusion were assessed using intracoronary ultrasound and Doppler, and coronary sinus and arterial blood sampling.

Results Intrabrachial substance P infusion was well tolerated and produced reproducible increases in forearm blood flow and tissue plasminogen activator release without affecting plasminogen activator inhibitor type 1 and von Willebrand factor concentrations. In contrast, endothelin-1 and L-monomethyl arginine infusion did not cause acute tissue plasminogen activator release. The response to substance P infusion appears to be dependent on the endothelial cell neurokinin type 1 receptor, and is, in part, mediated by the L-arginine:nitric oxide pathway. Whilst endothelium-dependent vasomotion was impaired in both cigarette smokers and patients with hypercholesterolaemia, tissue plasminogen activator release was diminished only in cigarette smokers and was unaffected by hypercholesterolaemia or lipid lowering therapy. Coronary fibrinolytic activity, but not endothelium-dependent coronary vasodilatation, inversely correlated with the volume of coronary artery atheroma.

Conclusions A model to assess the acute endogenous fibrinolytic capacity has been developed and characterised which was well tolerated and reproducible. Cigarette smoking, but not hypercholesterolaemia, is associated with an impairment of the acute tissue plasminogen activator release which may, in part, explain the increased propensity of smokers to sustain an acute myocardial infarction as well as to respond more favourably to thrombolytic therapy. The apparently normal fibrinolytic response in patients with hypercholesterolaemia indicates that endothelial dysfunction can be manifest in separate distinct pathways depending upon the nature of the insult. Finally, the demonstration of an association between the extent of coronary atheroma and the local endogenous fibrinolytic response provides a potentially important mechanism through which endothelial dysfunction can directly contribute to the thrombotic consequences of coronary artery disease.

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DECLARATION

This thesis represents research undertaken in the Clinical Pharmacology Unit and Research Centre at the Western General Hospital and the Department of Cardiology at the Royal Infirmary, Edinburgh. The substantial part of the work described has been my own and carried out during the period between 1996 and 2000 whilst I was a Clinical Lecturer in Cardiology. I have been fortunate in gaining the advice and assistance of many colleagues, and they have been formally acknowledged. The work has been published in peer reviewed journals: see Bibliography. The thesis has not been accepted in any previous applications for a degree and all sources of information have been acknowledged.

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ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
ANOVA	Analysis of variance
APV	Average peak velocity
AUC	Area under the curve
bFGF	Basic fibroblast growth factor
cAMP	Cyclic adenosine monophosphate
CBF	Coronary blood flow
CSA	Cross-sectional area
EDHF	Endothelium-derived hyperpolarising factor
EDTA	Ethylene diamine tetraacetic acid
ELAM-1	Endothelium-leucocyte adhesion molecule type 1
ET_A	Endothelin type A receptor
ET_B	Endothelin type B receptor
ET-1	Endothelin-1
FBF	Forearm blood flow
Hct	Haematocrit
HLA	Histocompatibility leucocyte antigen
ICAM-1	Intercellular adhesion molecule type 1
ID_{90}	Inhibitory dose to reduce response by 90%
IL	Interleukin
IVUS	Intravascular ultrasound
L-NMMA	L-N^G-monomethyl arginine
NK_1	Neurokinin type 1 receptor
PAI	Plasminogen activator inhibitor
PAF	Platelet activating factor
PD_{100}	Provocation dose to produce 100% vasodilatation
SEM	Standard error of the mean
SNP	Sodium nitroprusside
TNF-α	Tumour necrosis factor-alpha
t-PA	Tissue plasminogen activator
u-PA	Urinary-type plasminogen activator
VIP	Vasoactive intestinal peptide
VCAM-1	Vascular cellular adhesion molecule type 1
vWf	von Willebrand factor

CHAPTER 1

INTRODUCTION:

ENDOGENOUS FIBRINOLYSIS AND ENDOTHELIAL FUNCTION

1.1 THE ENDOGENOUS FIBRINOLYTIC SYSTEM

The fibrinolytic pathway describes a complex process involving the hydrolytic cleavage of fibrin, by plasmin, to cause clot dissolution and generate fibrin degradation products (see Figure 1.1). Plasmin, a serine protease, is generated by the action of an array of enzymes and inhibitors which have a co-ordinated action on the zymogen, plasminogen. The principal role of this enzymatic system is to protect the circulation from intravascular fibrin formation and thrombosis that would otherwise result in vessel occlusion and tissue ischaemia. However, it should also be acknowledged that in addition to its role in the degradation of intravascular thrombus, the fibrinolytic system is involved in many disparate physiological and pathophysiological processes including macrophage migration [Saksela & Rifkin 1988], ovulation [Strickland & Beers 1976], embryogenesis [Saksela & Holthofer 1987] and tumour invasion [Danø *et al* 1985].

1.1.1 Initiation Of Fibrinolysis

1.1.1.1 *Plasminogen And Plasmin*

Plasminogen is principally produced by the liver [Raum *et al* 1980] as a single chain glycoprotein containing 791 amino acids and has a plasma concentration of ~2 μmol/L in man. The gene for human plasminogen is encoded on chromosome 6q26-q27, encompasses 19 exons over 54 kb, and has some sequence homology with the nearby apolipoprotein (a) gene. The protein structure of plasminogen includes a preactivation peptide sequence, five kringle domains and a catalytic protease domain

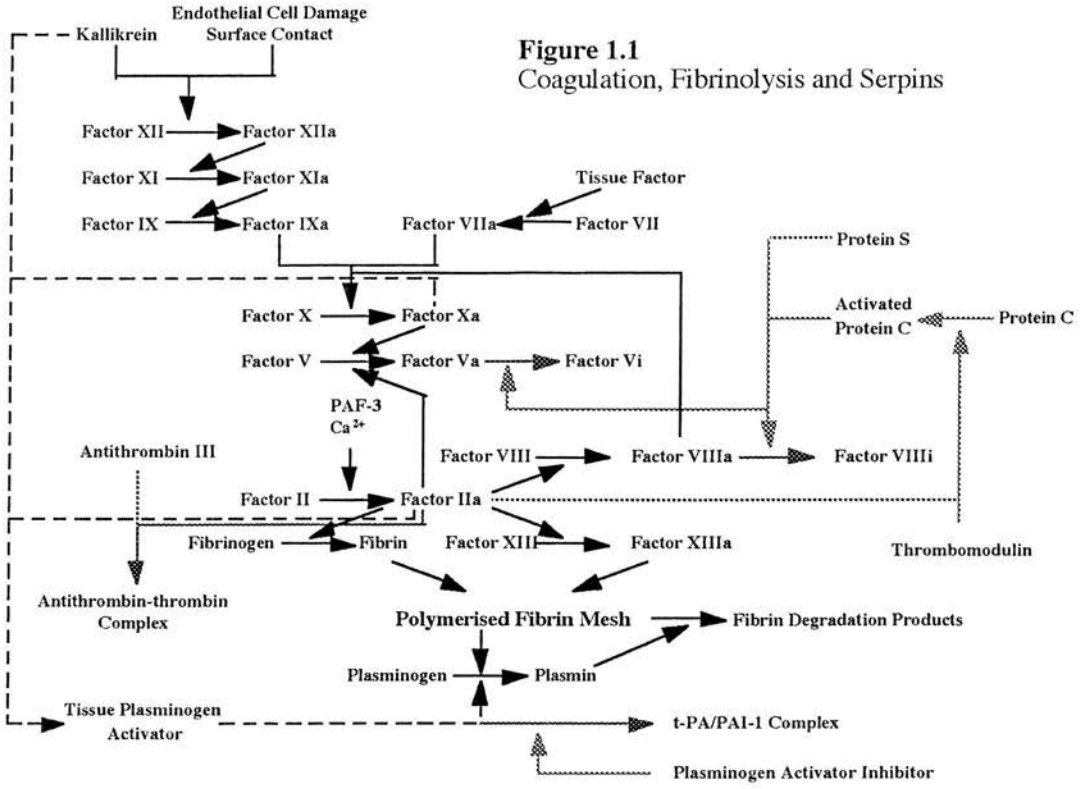


Figure 1.1
Coagulation, Fibrinolysis and Serpins

[Petersen *et al* 1990]. Kringle domains 1 and 5 are responsible for the binding of plasminogen to fibrin [Petersen & Suenson 1990, Wu *et al* 1990].

The conformational structure of plasminogen undergoes dramatic change when the preactivation peptide is removed by plasmin. Conversion of plasminogen to plasmin is achieved through the cleavage of the Arg^{561}-Val^{562} peptide bond by plasminogen activators, generating two polypeptide chains (60 and 25 amino acids) held together by a single disulphide bridge. The mature plasmin molecule is then able to degrade fibrin to generate various fibrin degradation products, such as D-dimers and fragment E [Gaffney *et al* 1976].

1.1.1.2 *Plasminogen Activators*

The initiation of fibrinolysis is dependent on the actions of plasminogen activators of which there are two endogenous forms: tissue plasminogen activator (t-PA) and urinary-type plasminogen activator (u-PA). The main physiological plasminogen activator involved in the removal of intravascular fibrin is t-PA [Åstedt 1979; Kok 1979]. In contrast, u-PA appears to have a role in facilitating cellular migration and, unlike t-PA, two chain u-PA lacks fibrin-specificity and will indiscriminately activate plasminogen.

1.1.2 Inhibition Of Fibrinolysis

Serine protease inhibitors, or serpins, represent a family of related regulatory peptides that have a major role in the control of coagulation, fibrinolysis and inflammation. The regulation of fibrinolysis by serpins principally occurs at two levels: inhibition of plasmin and inhibition of plasminogen activators.

1.1.2.1 *Plasmin Inhibitors*

Plasmin action is principally inhibited by α_2-antiplasmin although other factors, such as histidine-rich glycoproteins and thrombospondin, may play a minor role. α_2-Antiplasmin is a 452 amino acid glycoprotein [Holmes *et al* 1987] that acts as a high affinity, rapidly acting inhibitor of plasmin. Plasma concentrations of α_2-antiplasmin are ~1 μmol/L in man and consequently, even with extensive plasminogen activation, there is a sufficent reserve to inactivate liberated plasmin. Unbound free plasmin is rapidly complexed and inactivated by α_2-antiplasmin such that the indiscriminate

consumption of haemostatic factors, through fibrinogenolysis, is avoided [Wiman & Collén 1978]. However, fibrin bound plasmin is resistant to inactivation by α_2-antiplasmin since the binding site for α_2-antiplasmin is occupied by fibrin itself. This creates a mechanism whereby plasmin is only locally active at the site of thrombus formation.

1.1.2.2 *Plasminogen Activator Inhibitors*

There are several inhibitors of plasminogen activators but the main inhibitor of t-PA in man is plasminogen activator inhibitor type 1 (PAI-1). Other plasminogen activator inhibitors exist including PAI-2, PAI-3, α_2-macroglobulin and C1 esterase inhibitor. Originally isolated from placental tissue, PAI-2 appears to have a preferential inhibitory effect on u-PA suggesting that its primary role may be in regulating extracellular u-PA, such as during placental development.

1.2 TISSUE PLASMINOGEN ACTIVATOR

1.2.1 Protein Structure And Function

Human t-PA is principally produced by the endothelium as a glycoprotein containing 527 or 530 amino acids and has a plasma concentration of ~70 pmol/L. The gene for human t-PA is encoded on chromosome 8p12-q11.2 and comprises of 33 kb [Verheijen *et al* 1986]. Cleavage at Arg^{275} divides the t-PA molecule into two chains, A and B, which are held together by a single disulphide bridge. The protein structure of t-PA includes 5 main functional domains: finger, epidermal growth factor, kringle 1, kringle 2 and protease domains (see Figure 1.2). The finger and second kringle domains of chain A are responsible for fibrin binding whereas the protease domain of chain B contains the catalytic site which causes plasminogen activation through cleavage of the Arg^{561}-Val^{562} bond.

In the absence of fibrin, t-PA has very weak activity as a plasminogen activator. However, once bound to fibrin, the catalytic activity of t-PA rises a 1000-fold due to conformational changes resulting from the binding of the finger and second kringle domains [van Zonneveld *et al* 1986], and the formation of a ternary complex between plasminogen, fibrin and t-PA [Rånby 1982].

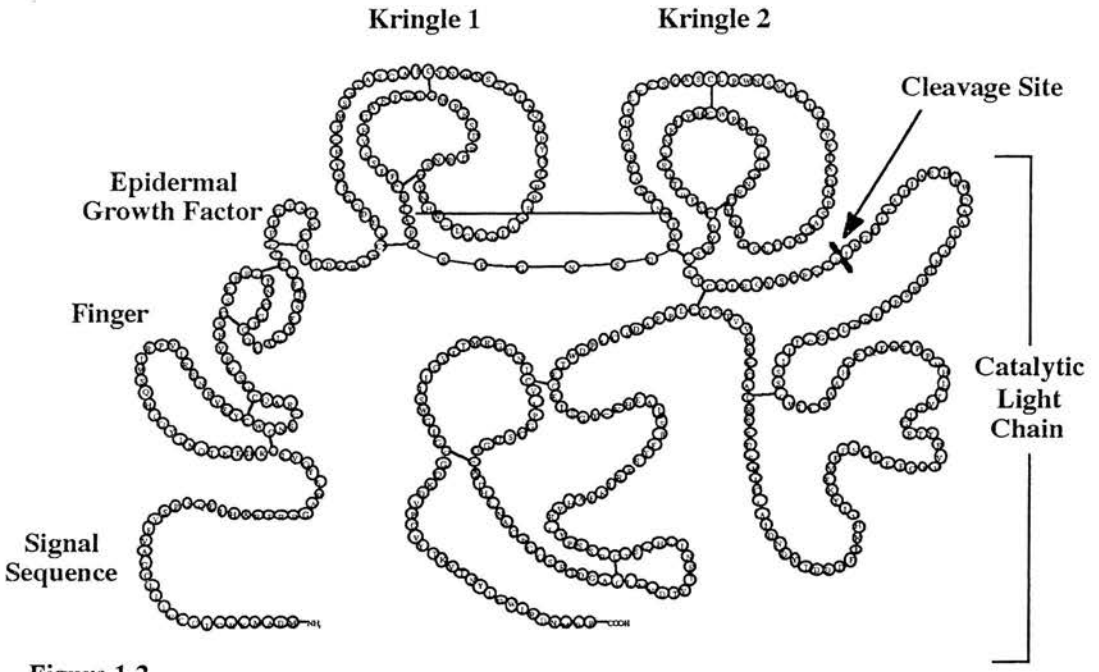


Figure 1.2
Protein structure of tissue plasminogen activator

1.2.2 Synthesis And Release

Although t-PA synthesis can be detected in mesothelial cells [van Hinsbergh *et al* 1990] and haematopoetic cells [Hart *et al* 1989], the endothelium is the principal site of its generation where it is released via both constitutive and regulated pathways. Endothelial cells synthesise, store and release t-PA in most of the blood vessels of man, and especially in the pre-capillary arterioles and post-capillary venules [Levin & del Zoppo 1994]. Regional differences in t-PA release do exist such that the upper limbs release four times the amount of the lower limbs [Keber 1988], and the liver and kidney do not acutely release t-PA when challenged with desmopressin [Brommer *et al* 1988].

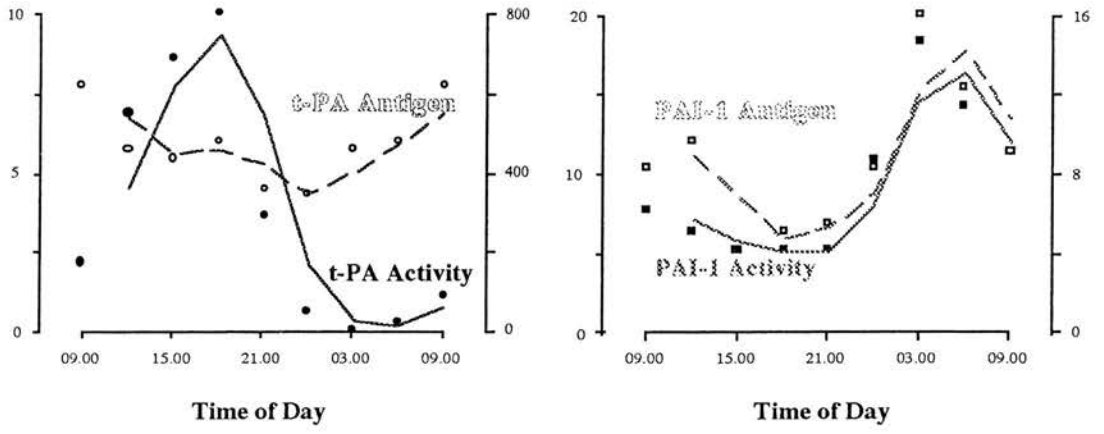
The release of t-PA may be rapidly increased through the translocation of a dynamic intracellular storage pool [van den Eijnden-Schrauwen *et al* 1995] in response to stimulation by blood coagulation and humoral factors [Emeis 1992]. This acute release does not require *de novo* protein synthesis since pretreatment with cycloheximide does not prevent t-PA release in the isolated rat hindlimb perfusion model [Tranquille & Emeis 1989]. Many physiological and pharmacological stimuli have been shown to release t-PA, some of which are listed in Table 1.1.

Table 1.1
Selection of factors modulating the fibrinolytic system and tissue plasminogen activator release.

Acetylcholine	Acidosis	Age
Adrenaline	bFGF	Alcohol
Bradykinin	Calcium	Coffee
Calcium	cAMP	Electroshock
Circadian Rhythm	Endothelin	Exercise
Histamine	Hyperoxia	Diet
Hypoxia	Insulin	Garlic
Interleukin-1	O_2 Radicals	Mental Stress
PAF	Prostaglandins	Onions
Substance P	Thrombin	Temperature
TNF-α	Vasopressin	Sex
VIP	Venous Occlusion	Smoking

There is a pronounced circadian variation in fibrinolytic activity with very low t-PA activity in the early morning [Andreotti & Kluft 1991]. Paradoxically, t-PA antigen concentrations peak early in the morning and the markedly reduced t-PA activity is a consequence of the four-fold rise in PAI-1 concentrations: see Figure 1.3.

Figure 1.3
Circadian rhythm [Andreotti & Kluft 1991]



1.2.3 Clearance Of T-PA And The t-PA/PAI-1 Complex

The clearance of t-PA predominantly occurs through the removal of both active t-PA and t-PA/PAI-1 complexes (see Figures 1.4 and 1.5) from the circulation by the liver [Chandler *et al* 1997]. This clearance process is rapid, with plasma half lives for active t-PA and t-PA/PAI-1 complexes of 2.4 and 5.0 minutes respectively. In the presence of high PAI-1 activity, more t-PA is complexed and the total clearance is slowed due to the longer half life of the t-PA/PAI-1 complex. The pharmacodynamics of t-PA clearance suggest a two compartment model [Chandler *et al* 1997] and it is likely that endothelial cell t-PA receptors contribute significantly to this effect [Cesarman *et al* 1994].

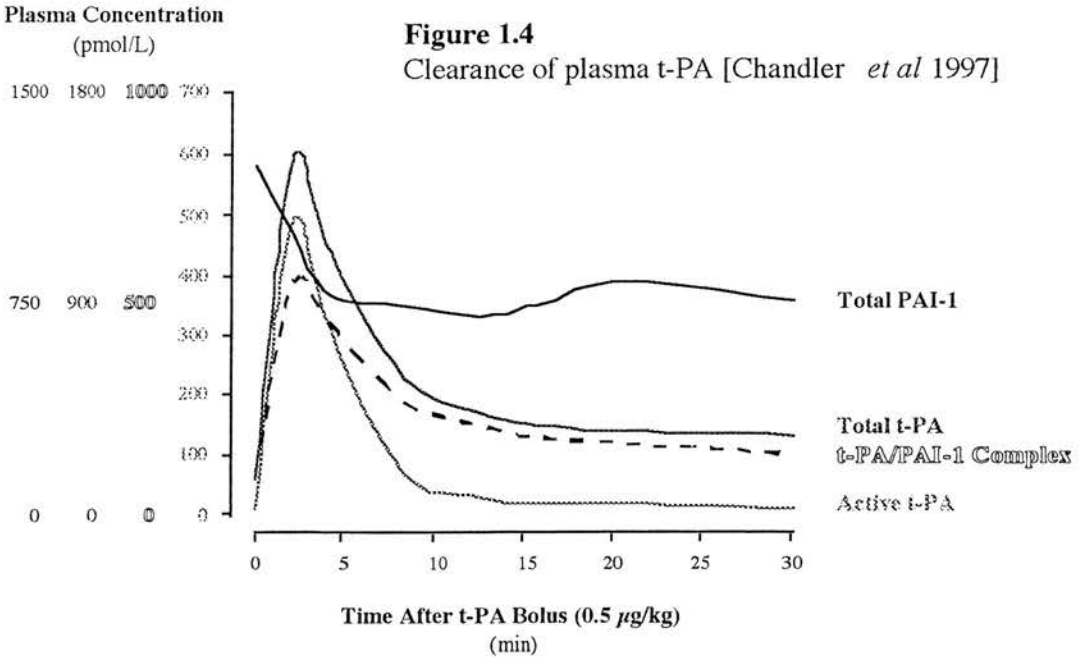
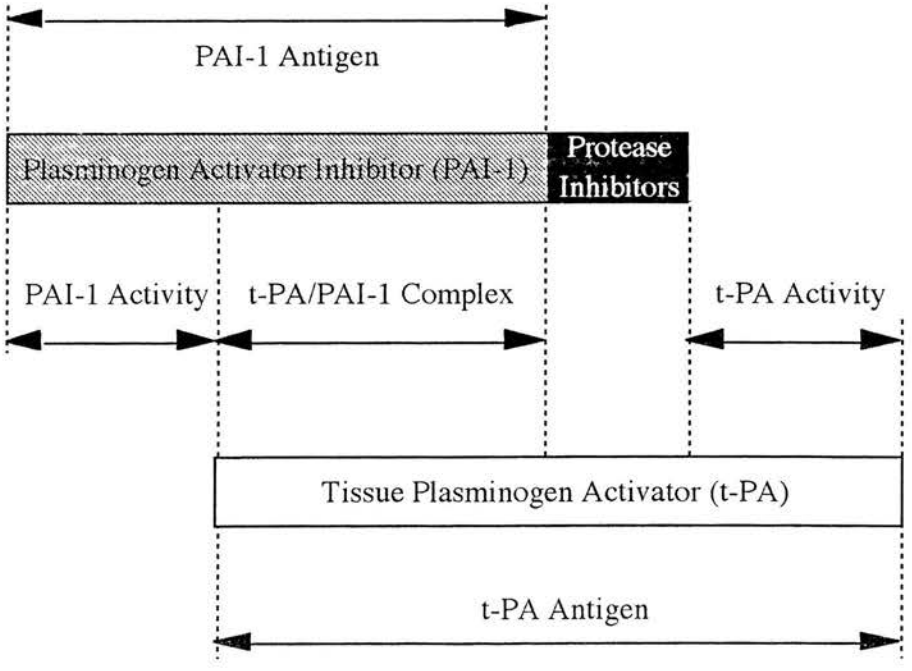


Figure 1.4
Clearance of plasma t-PA [Chandler *et al* 1997]

Figure 1.5
Relationship between t-PA and PAI-1 antigen and activity



1.3 PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1

1.3.1 Protein Structure And Function

Human PAI-1 is produced by several tissues [Sprengers *et al* 1986; Simpson *et al* 1991] including the endothelium, liver, platelets and vascular smooth muscle. It is a glycoprotein containing 379 amino acids and has a plasma concentration of ~500 pmol/L. The gene for human PAI-1 is located on chromosome 7q21.3-q22 and comprises of 12 kb encompassing 9 exons [Loskutoff *et al* 1987].

There are two conformational forms of PAI-1: an active and inactive form [Levin 1986]. The active form is unstable and conformational changes within the molecule cause PAI-1 to become inactive, the so-called "latent" form. Loss of activity occurs with a half life of 0.5-3 hours, although stability of the active PAI-1 form is enhanced by binding with vitronectin [Declerck *et al* 1988a]. PAI-1 has an Arg-Met reactive centre in keeping with other serpins, and binds to the kringle 2 domain of t-PA [Kaneko *et al* 1991]. This has the effect of preventing t-PA binding to fibrin and inhibiting its mode of action in a manner analogous to α_2-antiplasmin and plasmin.

1.3.2 Synthesis And Release

The origin of plasma PAI-1 remains unclear but does not appear to arise from platelets since plasma concentrations are normal in patients with thrombocytopenia and grey platelet syndrome [Booth *et al* 1988]. It would seem that the majority of PAI-1 synthesis and release occurs in vascular tissue, namely endothelium and

vascular smooth muscle [Simpson *et al* 1991], although the liver may provide a significant further contribution.

There are high concentrations of PAI-1 in the α-granules of platelets and this may be an important source of plasminogen activator inhibition during thrombus formation with platelet activation leading to high local concentrations of PAI-1. The activity of platelet-derived PAI-1 is only a fraction (5%) of plasma PAI-1, possibly due to the lack of the stabilising influence of vitronectin. However, because of the proportionately larger overall mass, platelet PAI-1 may account for some 50% of the overall total inhibitory activity of PAI-1 [Booth *et al* 1988].

1.4 ENDOTHELIUM

The endothelium plays a central role in the maintenance of normal vascular function and homeostasis including the control of blood flow, coagulation, fibrinolysis and inflammation. Consequently, the maintenance and regulation of tissue perfusion critically depends upon the integrity of endothelial function and the release of various endothelium-derived factors. Some of the major endothelial cell products are listed in Table 1.2.

Table 1.2
Endothelial cell derived mediators of vascular homeostasis

Vasomotion		
Nitric Oxide	Prostacyclin	EDHF
Endothelin	Angiotensin II	
Coagulation		
von Willebrand factor	Tissue factor	Thrombomodulin
Binding sites for: factors Va, IXa and Xa		Protein S
Tissue factor inhibitor		
Fibrinolysis		
Tissue plasminogen activator		
Plasminogen activator inhibitor		
Inflammation		
Selectin expression: E-selectin, P-selectin		
Leucocyte adhesion molecules: ICAM-1, VCAM-1, ELAM-1		
Cytokines: IL-1, IL-6, IL-8		
Major HLA antigens (class I and II)		
Growth factors		
Basic fibroblast growth factor		
Vascular endothelial growth factor		

1.4.1 Endothelium-Dependent Vasomotion

Since the seminal work of Furchgott and Zawadski [Furchgott & Zawadski 1980], it has been widely recognised that an array of mediators can influence vascular tone through endothelium-dependent actions [Furchgott 1984]. Endothelial stimulation results in the immediate release of a number of vasodilator mediators including prostacyclin, nitric oxide 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. Indeed, there appears to be an inextricable interplay between these endothelium-derived vasodilator and vasoconstrictor mediators [Verhaar *et al* 1998].

Both nitric oxide and endothelin-1 are released continuously by the endothelium to regulate basal vascular tone [Vallance *et al* 1989; Haynes & Webb 1994] and blood pressure [Haynes *et al* 1993; Haynes *et al* 1996]. Thus, the endothelium has a central role in the local paracrine regulation of vascular tone, blood flow and blood pressure. Disruption of normal endothelial function can, therefore, have a marked influence on the local and systemic haemodynamic balance, and ultimately tissue perfusion.

1.4.2 Endothelium-Derived Haemostatic Factors

The endothelium has an intrinsic anticoagulant action due to the presence of glycosaminoglycans, which are rich in heparan sulphates, in the cellular plasma membrane. These glycosaminoglycans bind antithrombin III and provide a surface that rapidly inactivates thrombin [Busch & Owen 1982]. In addition,

thrombomodulin, a cell surface glycoprotein, adds to the anticoagulant action of the endothelium by binding of thrombin and activating protein C [Esmon 1987].

Von Willebrand factor (vWf) is only produced by the endothelium and platelets, and is a cofactor which stabilises factor VIII:C and facilitates platelet adhesion to the subendothelial matrix. Approximately 90% of plasma vWf originates from the endothelium [Bowie *et al* 1986] where it is both constitutively secreted as well as stored within the Weibel Palade bodies [Mayadas & Wagner 1991]. However, there is controversy as to whether the majority of stored t-PA co-localises with vWf in the Weibel Palade bodies [Datta *et al* 1999; Rosnoblet *et al* 1999] or is contained within separate distinct granules [Emeis *et al* 1997].

1.4.3 Endothelium-Derived Fibrinolytic Factors

Endothelial cells constitutively secrete both t-PA and PAI-1 and the rate of release can be differentially affected by cytokines, coagulant factors and endotoxin. The synthesis and release of t-PA and PAI-1 has been reviewed above.

1.5 ENDOTHELIAL FUNCTION, ENDOGENOUS FIBRINOLYSIS AND ATHEROTHROMBOSIS

1.5.1 Endothelial Dysfunction And Atherosclerosis

The endothelium-dependent regulation of peripheral vascular tone is impaired by many of the risk factors associated with atherosclerosis, such as hypercholesterolaemia [Chowienczyk *et al* 1992; Stroes *et al* 1995; Heitzer *et al* 1996a], diabetes mellitus [Calver *et al* 1992], familial history of atherosclerosis [Celermajer *et al* 1992] and smoking [Celermajer *et al* 1996; Heitzer *et al* 1996a+b; Newby *et al* 1999a]. This dysfunction appears to be generalised with demonstrable impairment of endothelial function in the coronary circulation of patients with coronary artery disease [Ludmer *et al* 1986] and its risk factors [Vita *et al* 1990] including smoking [Zeiher *et al* 1995].

The formation of atherosclerosis is a complex inflammatory process that involves a series of interactions with many cell types including the endothelium [Ross 1999]. To date, the clinical dynamic assessment of endothelial dysfunction has predominantly relied upon the measurement of endothelium-dependent vasomotion. This has been undertaken by determining resistance vessel responses to endothelial cell stimulants [Ludmer *et al* 1986; Chowienczyk *et al* 1992; Stroes *et al* 1995] and nitric oxide synthase inhibition [Calver *et al* 1992], and conduit vessel responses to reactive hyperaemia [Celermajer *et al* 1996] - so called flow associated dilatation. However, whilst providing an important indicator of endothelial function, endothelium-dependent vasomotion can be viewed as an indirect surrogate measure

of the main pathophysiological role of the endothelium in atherothrombosis. In contrast, t-PA is a potentially important endothelium-derived mediator that is intimately linked to the risk of thrombosis.

1.5.2 Ischaemic Heart Disease And Endogenous Fibrinolysis

In epidemiological studies of patients with ischaemic heart disease [Hamsten *et al* 1985; Thompson *et al* 1995], and in prospective studies in healthy populations [Ridker *et al* 1993a; Thögersen *et al* 1998], higher total plasma t-PA (antigen) concentrations positively and independently predict future coronary events. It would be anticipated, however, that high t-PA concentrations would protect against subsequent coronary events rather than the reverse. This paradoxical association is, in part, explained by the concomitant elevation of PAI-1 which complexes with t-PA and, therefore, causes an overall reduction in free t-PA 'activity' [Jansson *et al* 1993; De Bono 1994]. It is this free and unbound t-PA which is physiologically active and central to endogenous fibrinolysis. Moreover, the time course of endogenous t-PA release is important, with thrombus dissolution being much more effective if t-PA is incorporated during, rather than after, thrombus formation [Brommer 1984; Fox *et al* 1984]. However, the capacity of endothelial cells to release t-PA from intracellular storage pools, and the rapidity with which this can be mobilised, may not be reflected in the basal circulating plasma concentrations of t-PA antigen or activity [Jern *et al* 1999].

1.5.3 Dynamic Assessment Of Acute Tissue Plasminogen Activator Release

The dynamic assessment of endogenous fibrinolysis has previously relied on the acute release of t-PA in response to venous occlusion, systemic desmopressin infusion or exercise [Allen *et al* 1985; Gris JC *et al* 1991; Held *et al* 1997; Jahun-Vague *et al* 1996]. However, the venous occlusion response is variable and has poor intra-individual reproducibility [Sultan *et al* 1988] and gives only a relatively crude measure of fibrinolytic capacity. In contrast, systemic desmopressin infusion appears to provide a more predictable fibrinolytic response in healthy volunteers [Prowse *et al* 1984] and patients with coronary artery disease [Duprez *et al* 1991]. However, desmopressin also causes marked vasodilatation and, when administered systemically, leads to facial flushing, an increase in heart rate and a fall in blood pressure. It has been hypothesised that the consequent systemic neurohumoral response to desmopressin induced vasodilatation may contribute to, or even mediate, the haemostatic and fibrinolytic response to desmopressin infusion [Grant *et al* 1988]. Moreover, *in vitro* endothelial cell culture studies have suggested that desmopressin at very high concentrations induces endothelial vWf release by a mechanism which is dependent on the presence of peripheral blood monocytes [Hashemi *et al* 1990], possibly mediated by the production of a second messenger molecule [Hashemi *et al* 1993]. Finally, we have recently shown that, in healthy male volunteers, desmopressin releases t-PA, vWf and Factor VIII:C predominantly via systemic mechanisms, possibly mediated by cytokine release [Newby *et al* 2000a]. There is, therefore, a need for a specific, reproducible and direct endothelium-dependent method of assessing the capacity to release t-PA acutely *in vivo* in man.

1.5.4 Tissue Plasminogen Activator Release In The Coronary Circulation

Acute rupture or erosion of a coronary atheromatous plaque and subsequent coronary artery thrombosis causes the majority of sudden cardiac deaths and myocardial infarctions [Burke *et al* 1997]. Small areas of denudation and thrombus deposition are a common finding on the surface of atheromatous plaques and are usually sub-clinical [Davies *et al* 1988]. However, in the presence of an imbalance in the fibrinolytic system, such microthrombi may propagate, ultimately leading to arterial occlusion [Rosenberg & Aird 1999]. The importance of local fibrinolysis and acute endogenous t-PA release is exemplified by the high rate of spontaneous reperfusion in the infarct related artery after acute myocardial infarction; occurring in around one third of patients within the first 12 hours [DeWood *et al* 1980; Armstrong *et al* 1989; Rentrop *et al* 1989].

In the Northwick Park Heart Study [Meade *et al* 1993], low basal plasma fibrinolytic activity was a leading determinant of the risk of sustaining a myocardial infarction or sudden cardiac death in younger men. Moreover, a reduction in exercise induced release of t-PA is associated with an increased incidence of major adverse cardiac events in patients with stable angina pectoris [Held *et al* 1997]. Recently, Rosenberg and Aird [Rosenberg & Aird 1999] have postulated that vascular-bed-specific defects in haemostasis exist, and that coronary thrombosis critically depends on the local fibrinolytic balance. However, there have been no clinical studies to date which have directly assessed the dynamic local fibrinolytic capacity of the coronary vascular bed in patients with coronary artery disease.

1.6 HYPOTHESES

Intra-brachial substance P has been previously shown to induce local fibrinolysis in the human forearm [Fanciullacci *et al* 1993] although the mechanism of this effect has not been determined. It is likely, however, that this effect is mediated through acute t-PA release but this suggestion awaits confirmation. Given that t-PA and vWf may co-localise within the endothelium [Datta *et al* 1999; Rosnoblet *et al* 1999] and that previous agents co-release both t-PA and vWf [Mannucci *et al* 1975; Ludlam *et al* 1980; Mannucci 1997], substance P may also cause acute release of vWf. This provides the basis for a potential model to assess not only endothelium-dependent vasomotion but also endothelium-dependent regulation of fibrinolysis and coagulation.

After initial development and characterisation of a model to assess endothelium-dependent vasomotion and endogenous t-PA release, the following hypotheses will be addressed:

1. The vascular actions of substance P are dependent on the neurokinin type 1 receptor and are, in part, mediated by the L-arginine:nitric oxide pathway.
2. Endothelium-independent vasodilators do not cause acute release of t-PA, PAI-1 and vWf in the forearm of man.
3. The acute release of endogenous t-PA is impaired in endothelial dysfunctional states, such as cigarette smoking and hyperlipidaemia.

4. Intra-coronary substance P causes the acute release of t-PA and PAI-1 in the coronary vascular bed.
5. The extent of coronary arterial atheroma will influence the acute local coronary release of t-PA.

1.7 AIMS

The aims of the thesis were:

In healthy male volunteers (Chapter 3):

- to establish the pharmacodynamics, tolerability and reproducibility of intra-arterial substance P, an endothelium- dependent vasodilator, in the forearm.

In healthy male volunteers (Chapter 4):

- to determine the ability of L-754,030, a neurokinin type 1 receptor antagonist, to inhibit substance P induced vasodilatation during and 24 hours after intravenous administration of its prodrug, L-758,298.
- to confirm that substance P induced vasodilatation is mediated via the endothelial cell NK_1 receptor in man.
- to determine whether endogenous substance P regulates peripheral vascular tone or blood pressure in man.
- to evaluate the safety and tolerability of single intravenous doses of L-758,298 in healthy male volunteers.

In healthy male volunteers (Chapter 5):

- to establish a method of determining the acute fibrinolytic capacity of the endothelium.

- to assess the acute release of coagulation and fibrinolytic factors within the forearm vascular bed in response to intra-arterial substance P using an ascending dose design to define the dose at which systemic effects intervene.
- to assess the potential release of coagulation and fibrinolytic factors in response to locally active doses of substance P and a control endothelium-independent nitric oxide donor, sodium nitroprusside.

In healthy male volunteers (Chapter 6):

- to determine whether endothelin-1, of exogenous or endogenous origin, acts via the endothelial ET_B receptor to regulate the release of t-PA, PAI-1 or vWf *in vivo* in man using synthetic endothelin-1 peptide and the selective ET_B receptor antagonist, BQ-788.

In healthy male volunteers (Chapter 7):

- to determine if prolonged substance P infusion can cause sustained t-PA release in the forearm.
- to ascertain whether prolonged substance P infusion can cause vWf or PAI-1 release.
- to determine whether nitric oxide synthase inhibition using L-N^G-monomethylarginine (L-NMMA) affects basal or substance P induced t-PA release.

In otherwise healthy volunteers (Chapter 8):

- to compare substance P induced t-PA release from the forearm vascular bed of smokers and age- and sex-matched non-smokers.

In patients with hypercholesterolaemia (Chapter 9):

- to determine whether there is an impairment of acute t-PA release .
- to ascertain if pre-treatment with pravastatin, a lipid lowering agent, could enhance t-PA release in these patients.
- to determine if substance P induced t-PA release has acceptable within subject reproducibility.

In patients with coronary artery disease (Chapter 10):

- to establish a model of assessing the dynamic local fibrinolytic capacity of the coronary vascular bed.
- to determine the relationship between the extent of coronary atheroma, as assessed by intravascular ultrasound, and the acute fibrinolytic capacity of the coronary circulation.

CHAPTER 2

METHODOLOGY:

MEASUREMENT OF FOREARM AND CORONARY BLOOD FLOW,

CORONARY PLAQUE VOLUME

AND

PLASMA FIBRINOLYTIC AND HAEMOSTATIC PARAMETERS

2.1 INTRODUCTION

When examining *in vivo* vascular responses in man, systemic drug administration causes concomitant effects on organs, such as the brain, kidney and heart, and influences neurohumoral reflexes through changes in systemic haemodynamics. Because of these confounding influences, vascular responses cannot be wholly attributed to a direct effect of the drug [Webb 1995]. Regional forearm and coronary infusions combined with bilateral venous occlusion plethysmography and intracoronary ultrasound provide methods of directly assessing peripheral and coronary vascular responses without invoking these systemic influences.

2.1.1 Forearm Resistance Vessels

Bilateral forearm blood flow measurements using venous occlusion plethysmography, with unilateral brachial artery infusion of vasoactive drugs at subsystemic, locally active doses, provides a powerful and reproducible method of directly assessing vascular responses *in vivo* [Benjamin *et al* 1995; Webb 1995]. This technique has been utilised very successfully to demonstrate the major contribution of nitric oxide and endothelin-1 to the maintenance of basal peripheral vascular tone in healthy man [Vallance *et al* 1989; Haynes *et al* 1994] and to predict that systemic inhibition of these systems would increase [Haynes *et al* 1993] and decrease [Haynes *et al* 1996] blood pressure respectively.

2.1.2 Coronary Circulation

The explosion in catheter based technology has put many innovative techniques at the disposal of the clinical investigator. Some of these techniques, such as the measurement of coronary and fractional flow reserves, have also found a role in clinical practice [White 1993] as a means of assessing the functional severity of intermediate coronary artery stenoses and thereby guide coronary intervention [Segal 1993]. Moreover, the advent of intravascular ultrasound, in particular, has facilitated the more detailed functional and morphological assessment of the coronary circulation.

Regional intracoronary infusions have the advantage of assessing the heart and coronary circulation in relative isolation without invoking systemic effects. This is particularly important for the assessment of cardiac and coronary function which is heavily dependent on changes in the systemic vasculature and haemodynamics. In addition, relatively high doses can be administered locally which may be important for the desired physiological or therapeutic effect and may be further facilitated by the use of local drug delivery systems. Finally, combining intracoronary drug administration with coronary sinus catheterisation and sampling can further extend the assessment of the coronary circulation to include additional aspects of cardiac metabolism and function.

2.2 GENERAL

2.2.1 Ethical Considerations

All studies were undertaken in accordance with the Declaration of Helsinki of the World Medical Association and with the approval of the Lothian Research Ethics Committee. The written informed consent of each subject or patient was obtained before entry into the study.

2.2.2 Subject Preparation

None of the normal healthy volunteers and controls 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 five hours before each study. Studies were performed in a quiet, temperature controlled room maintained at 23.5 - 24.5°C.

2.2.3 Blood Pressure Measurement

During forearm venous occlusion plethysmography, 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) [Wiinberg *et al* 1988].

2.3 FOREARM VENOUS OCCLUSION PLETHYSMOGRAPHY

2.3.1 Brachial Artery Cannulation

The brachial artery of the non-dominant arm was cannulated with a 27-G steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under 1% 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 P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 mL/min.

2.3.2 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 [Webb 1995]. 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 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 strain gauge

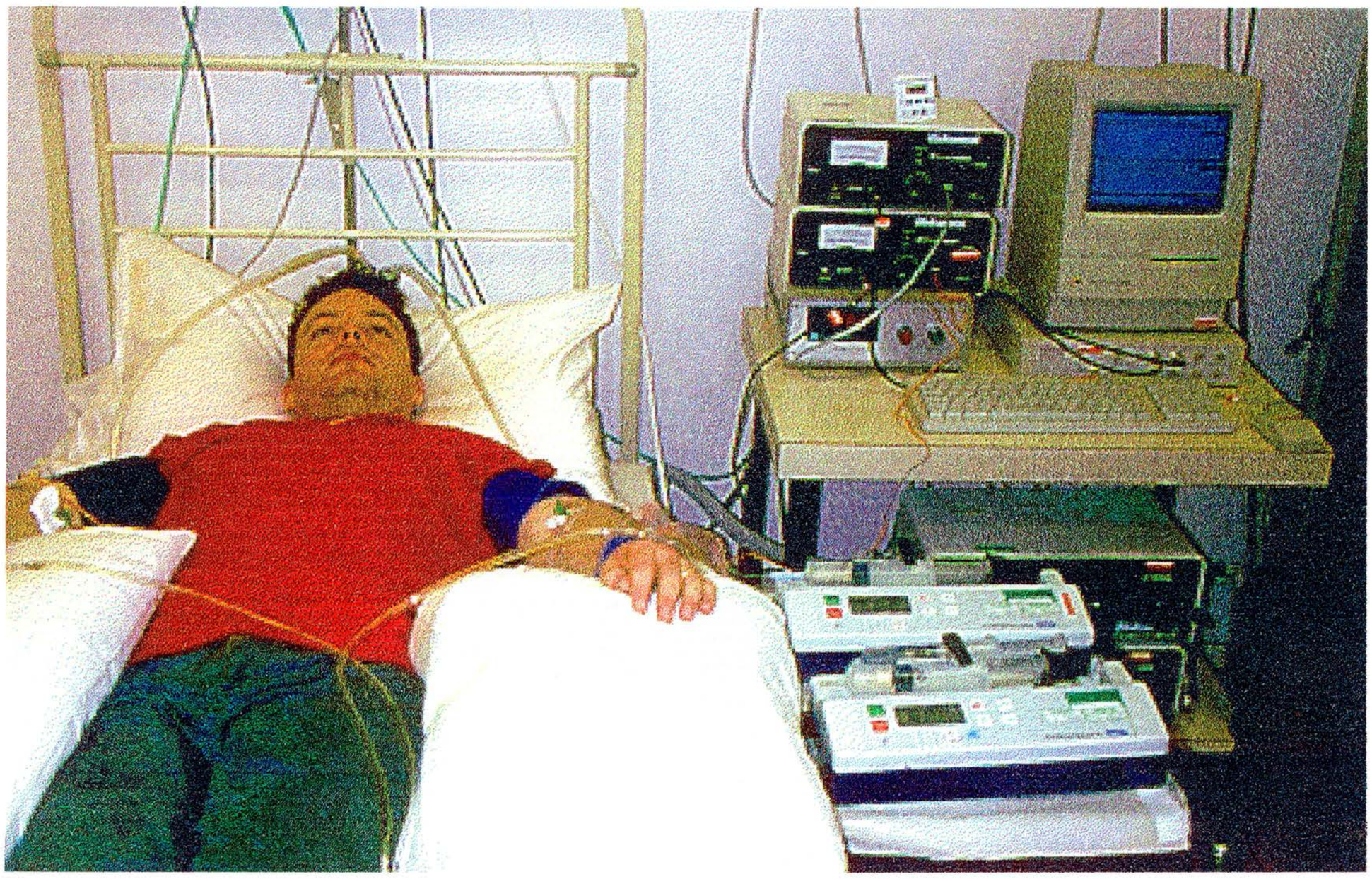


Figure 2.1

Forearm venous occlusion plethysmography: overall set up.

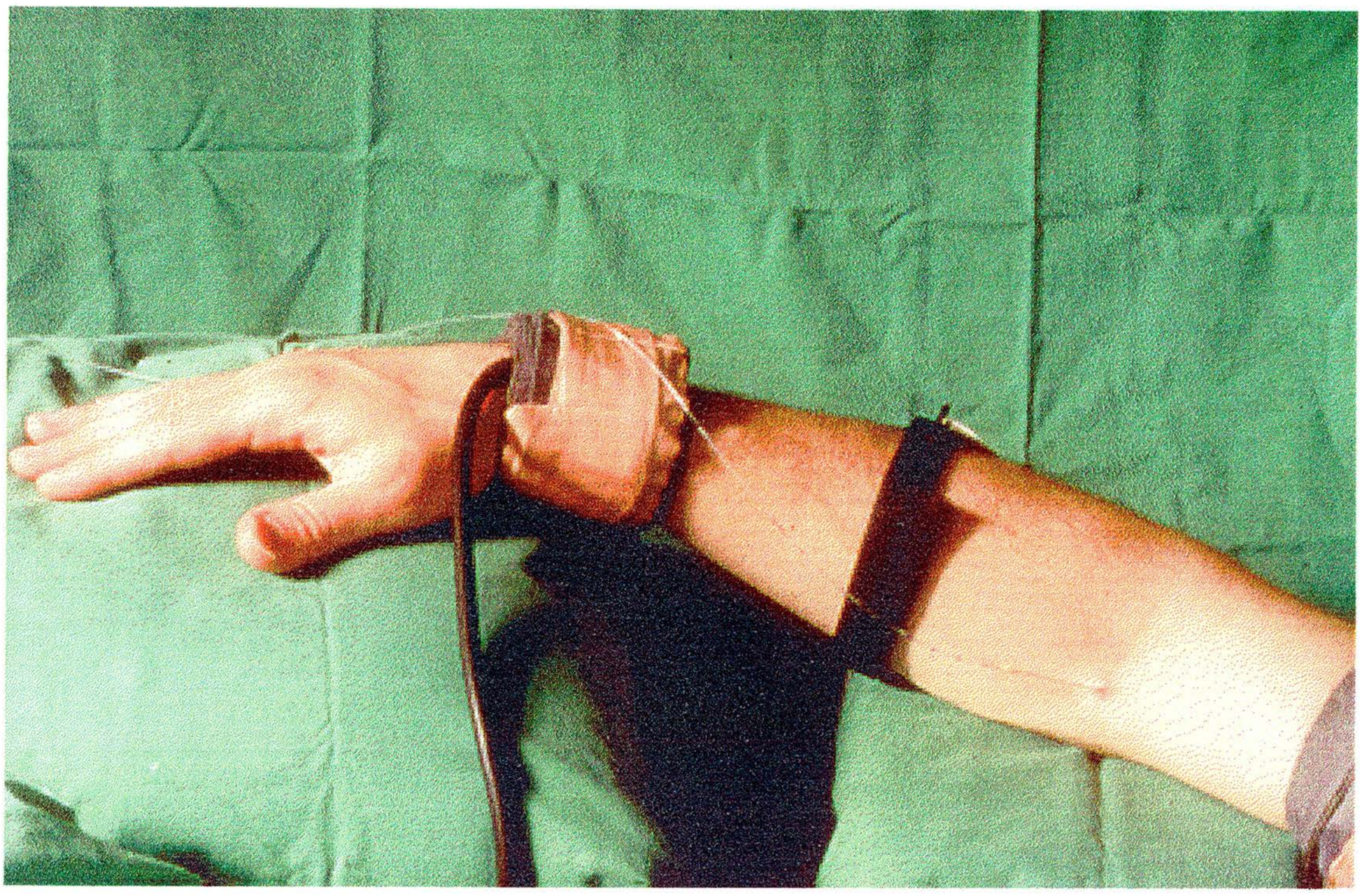


Figure 2.2

Forearm venous occlusion plethysmography: brachial artery cannulation.

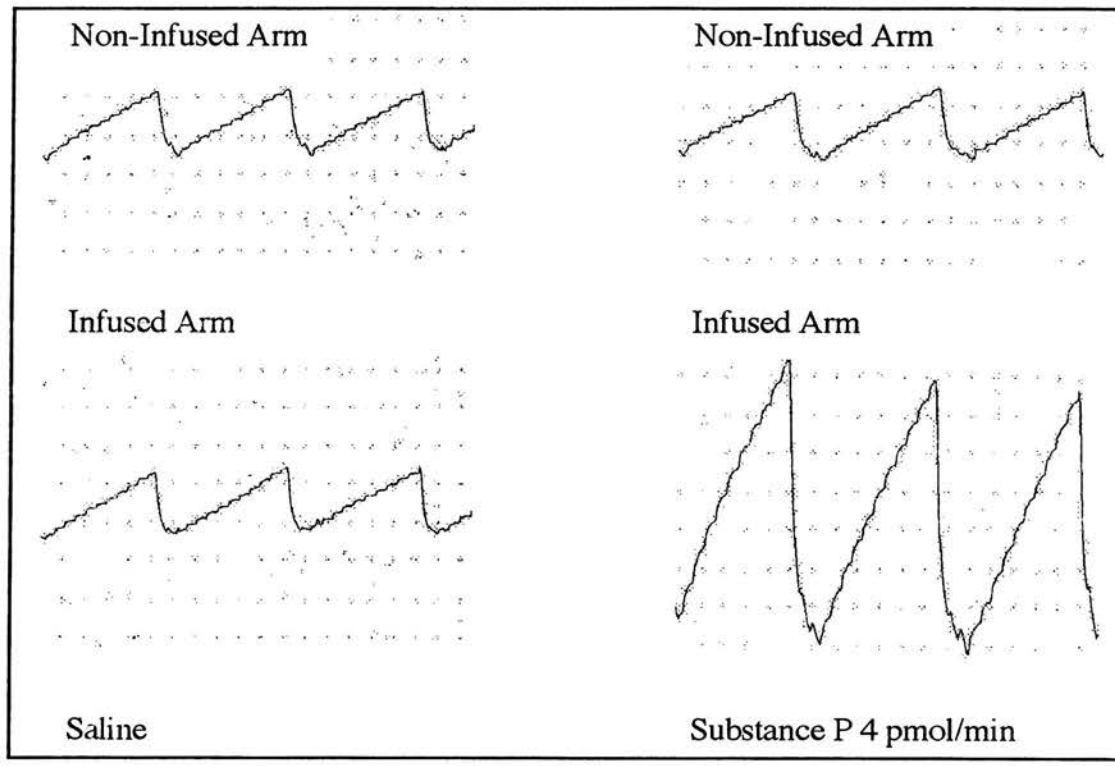


Figure 2.3

Forearm venous occlusion plethysmography: typical recording.

plethysmograph (D.E. Hokanson) was processed by a MacLab® analogue-to-digital converter and Chart™ *v*3.3.8 software (AD Instruments Ltd, Castle Hill, Australia) and recorded onto a Macintosh Classic II computer (Apple Computers Inc, Cupertino, USA). Calibration was achieved using the internal standard of the plethysmograph.

2.3.3 Data Analysis

Plethysmographic data were extracted from the Chart™ data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel *v*5.0; Microsoft). Recordings from the first 60 seconds after wrist cuff inflation were not used because of the variability in blood flow that this incurs [Kerslake 1949; Webb 1995]. Usually, the last five flow recordings in each three minute measurement period were calculated and averaged for each arm. To reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point: in effect using the non-infused arm as a contemporaneous control for the infused arm [Benjamin *et al* 1995; Webb 1995]. Percentage changes in the infused forearm blood flow were calculated [Benjamin *et al* 1995; Webb 1995] as follows:

$$\% \text{ Change in blood flow} = 100 \times \{I_t/NI_t - I_b/NI_b\} / I_b/NI_b$$

where I_b and NI_b are the infused and non-infused forearm blood flows at baseline (time 0) respectively, and I_t and NI_t are the infused and non-infused forearm blood flows at a given time point respectively.

2.4 INTRACORONARY ULTRASOUND

2.4.1 Left Coronary Artery Cannulation

Following diagnostic coronary angiography, patients with left main stem disease or a minimal luminal diameter of <2 mm in the left anterior descending artery were excluded because of the potential of the imaging catheter to impede anterograde coronary blood flow. All patients received 5,000 IU of intravenous heparin and, through an 8 F haemostatic sheath (Cordis®, Cordis Europa N.V., Roden, The Netherlands), the left coronary artery was cannulated with a 7 F guide catheter (Cordis®).

2.4.2 Morphometric Assessment: Intravascular Ultrasound (IVUS)

Following insertion of the 0.014 inch Doppler guide wire, 3.2 F Ultracross™ IVUS imaging catheter (SCIMED®, Boston Scientific Corporation, MN, USA) was advanced into left anterior descending coronary artery over the guide wire. The imaging probe was retracted within the imaging catheter sheath at 0.5 mm/s using a motorised pullback device (Boston Scientific Corp). Ultrasound images were recorded onto S-VHS tape using the Clearview™ ultrasonogram (Boston Scientific Corp).

Computerised three-dimensional reconstructions of the proximal left anterior descending coronary artery were performed off-line by a single blinded operator using the TomTec computer system (Echoscan, TomTec Imaging Systems, Unterschleissheim, Germany). The proximal atheromatous plaque volume was

calculated using a well validated edge detection algorithm [von Birgelen *et al* 1996a; von Birgelen *et al* 1997]. The planar contours were checked by the operator and, if necessary, edited. The plaque area was defined as the region between the luminal border and the echogenic interface of the external elastic lamina as previously described [Di Mario *et al* 1998; von Birgelen *et al* 1996b].

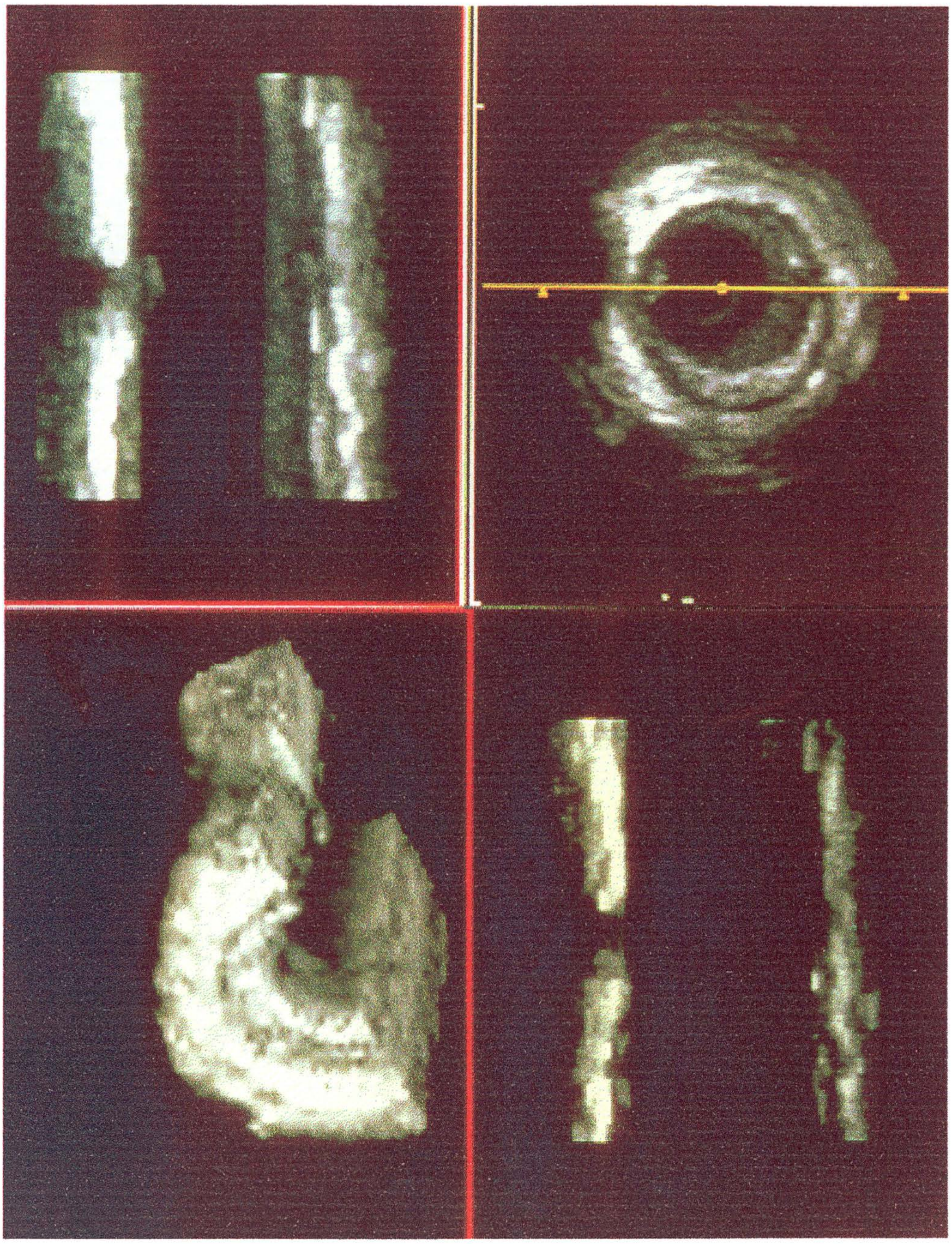


Figure 2.4

Intravascular ultrasound: cross-sectional views and three dimensional reconstruction in a patient with coronary atheroma.

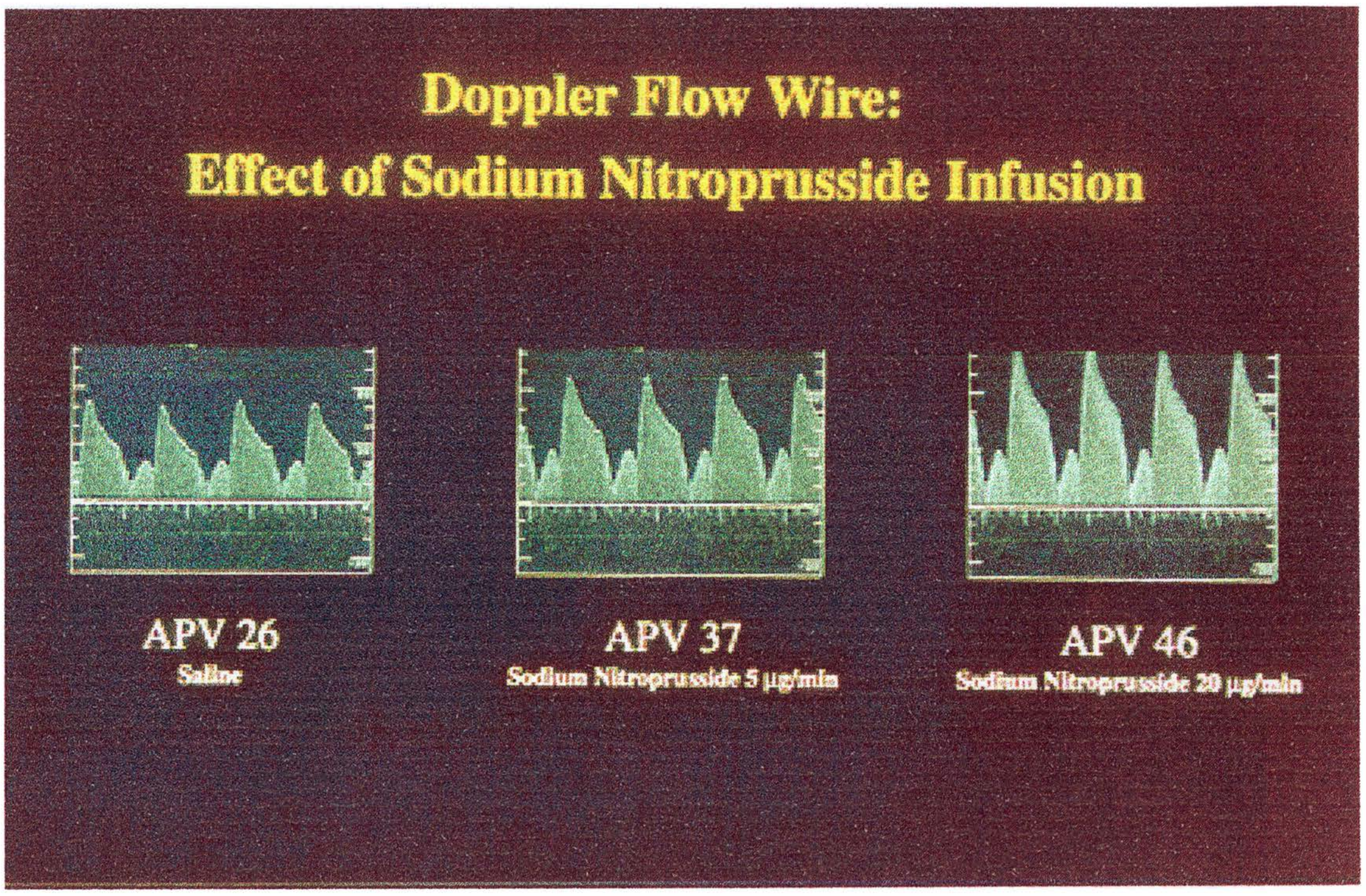


Figure 2.5

Doppler wire measurement of coronary flow velocity.

2.4.3 Intracoronary Drug Infusion

Selective left anterior descending coronary artery drug infusion was attained by infusion through the flush port of the IVUS catheter [Schwarzacher *et al* 2000]. All drugs were dissolved in saline (0.9%: Baxter Healthcare Ltd, Thetford, UK) and infused via an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intracoronary infusions was maintained constant throughout all studies at 1 mL/min.

2.4.4 Coronary Blood Flow Measurement

Following recording of the IVUS pullback examination, the IVUS imaging catheter was repositioned at the ostium of the left anterior descending artery just distal to the bifurcation of the left main stem. The Doppler guide wire (0.014 inch Flowire™, Cardiometrics, Endosonics, Rancho Cordova, CA) was retracted to the tip of the imaging sheath. The average peak velocity of the Doppler signal was recorded onto S-VHS tape using the Flomap™ (Cardiometrics, Endosonics, Rancho Cordova, CA) Doppler velocimeter.

The left anterior descending artery cross-sectional area was measured using computerised planimetry (Clearview™, Boston Scientific Inc) of the IVUS vessel luminal area. The images were gated to the electrocardiogram and measurements were made at the onset of the QRS complex. Blood flow velocity was determined using average peak velocity of the Doppler signal (Flopmap™ (Cardiometrics, Endosonics). Blood flow in the left anterior descending coronary artery was defined as [Doucette *et al* 1992]:

$$\text{Coronary blood flow (mL/min)} = \text{CSA} \cdot \frac{\text{APV}}{2} \cdot \frac{60}{100}$$

where CSA = cross-sectional area (mm^2) and APV = average peak velocity (cm/s).

2.5 FIBRINOLYTIC AND HAEMOSTATIC PARAMETERS

2.5.1 Forearm Venous Sampling

Venous cannulae (17G) were inserted into large subcutaneous veins of the antecubital fossa in both arms as described previously [Plumpton *et al* 1995]. Ten mL of blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool® Stabilyte™, Umeå, Sweden) and citrate (Monovette®, Sarstedt, Nümbrecht, Germany) tubes, and kept on ice before being centrifuged at 2,000 *g* for 30 minutes at 4°C. Platelet free plasma was decanted and stored at -80°C before assay [Kluft & Verheijen 1990].

2.5.2 Arterial And Coronary Sinus Blood Sampling

Arterial samples were obtained through an 8 F haemostatic sheath placed in the right femoral artery. Cannulation of the coronary sinus from the femoral vein was performed using a preformed specific 6F catheter (modified Simmons Torcon NB catheter, HNB6.0-NT-100-PW-2S-112393-BH) [Katritsis & Webb-Peploe 1997]. To avoid atrial blood mixing, the catheter was advanced, sometimes with the assistance of a guide wire, deep into the ostium and beyond the posterior interventricular vein. Adequate positioning of the catheter was ensured by determining coronary sinus blood oxygen saturations using an automated oximeter (Oxicam™ 300, Watco Services, Basingstoke, UK).

2.5.3 Plasma Fibrinolytic Parameter Assays

Plasma PAI-1 and t-PA antigen concentrations were determined using an enzyme-linked immunosorbent assay; Coaliza® PAI-1 [Declerck *et al* 1988b] and Coaliza® t-PA [Booth *et al* 1987] (Chromogenix AB, Mölndal, Sweden) respectively. Plasma PAI-1 and t-PA activities were determined by a photometric method, Coatest® PAI-1 [Wiman *et al* 1988] and Coaset® t-PA [Gram *et al* 1987] (Chromogenix AB). 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.

2.5.4 Plasma Haemostatic Factor Assays

Von Willebrand factor (vWf) antigen was determined [Cejka 1982] using an enzyme-linked immunosorbent assay (Dako A/S, Glostrup, Denmark) with a sensitivity of 0.05 IU/mL. The intra-assay and inter-assay coefficients of variability were 5.2% and 7.3% respectively. Factor VIII:C procoagulant activity was determined using a standard one stage assay on an ACL-3000+ coagulometer (Instrumentation Laboratory, Warrington, UK).

2.5.5 Haematocrit Measurement

Haematocrit was determined by capillary tube centrifugation of blood anticoagulated by ethylene diamine tetraacetic acid.

2.5.6 Data Analysis And Statistics

For the forearm studies, estimated net release of t-PA activity and antigen was defined as the product of the infused forearm plasma flow (based on the haematocrit, Hct and the infused forearm blood flow, FBF) and the concentration difference between the infused ($[t\text{-}PA]_{Inf}$) and non-infused arms ($[t\text{-}PA]_{Non\text{-}inf}$).

$$\text{Estimated net forearm t-PA release} = FBF \times \{1\text{-}Hct\} \times \{[t\text{-}PA]_{Inf} - [t\text{-}PA]_{Non\text{-}inf}\}$$

Because the coronary sinus drains some regions of the heart outwith the left anterior descending coronary artery territory, the net release of t-PA will tend to be under estimated. However, an index of cardiac t-PA release was defined as the product of the left anterior descending artery plasma flow (based on the haematocrit, Hct and the left anterior descending coronary blood flow, CBF) and the plasma arterial ($[t\text{-}PA]_{Art}$) and coronary sinus ($[t\text{-}PA]_{Ven}$) concentration differences.

$$\text{Estimated net cardiac t-PA release} = CBF \times \{1\text{-}Hct\} \times \{[t\text{-}PA]_{Ven} - [t\text{-}PA]_{Art}\}$$

Data were examined by analysis of variance (ANOVA) with repeated measures, two tailed paired Student's *t*-test and regression analysis using Excel *v*5.0 (Microsoft). All results are expressed as mean ± standard error of the mean. Statistical significance was taken at the 5% level.

CHAPTER 3

INTRA-ARTERIAL SUBSTANCE P MEDIATED VASODILATATION IN THE HUMAN FOREARM: PHARMACOLOGY, REPRODUCIBILITY AND TOLERABILITY

Newby DE, Sciberras DG, Mendel CM, Gertz BJ, Boon NA, Webb DJ.
Intra-arterial substance P mediated vasodilatation in the human forearm: pharmacology, reproducibility and tolerability.
Br J Clin Pharmacol 1997;**43**:493-499.

3.1 SUMMARY

The current studies were designed to characterise the pharmacology, reproducibility and tolerability of the vasodilator response to intra-arterial substance P infusion in the forearm of healthy man. On different occasions, eight healthy male volunteers received brachial artery infusions of substance P at doubling doses ranging from 0.5 to 128 pmol/min. Blood flow was measured in both arms using venous occlusion plethysmography. Substance P induced dose-dependent vasodilatation in the human forearm which had a log-linear relationship to dose. At doses of 1 to 8 pmol/min, mean responses were highly reproducible both within and between days. There were no differences between responses to discontinuous doses and continuous doses of substance P. Substance P was generally well tolerated at doses of ≥64 pmol/min with no significant alteration in arterial blood pressure or heart rate. Skin oedema in the infused forearm and systemic vasodilatation, manifested by facial flushing and non-infused forearm vasodilatation, occurred at doses of ≥16 pmol/min. Forearm vasodilatation to substance P represents a reproducible and useful model in the assessment of peripheral endothelial cell NK_1 receptor function.

3.2 INTRODUCTION

Substance P is a widely distributed endecapeptide which is found principally in the neural tissue of the central, peripheral and enteric nervous systems [Hökflet *et al* 1977; Lundberg *et al* 1979; Pernow 1983; Aronin *et al* 1986]. The physiological functions of substance P include neurotransmission in primary sensory neurones, with particular involvement in nociception and emesis. In addition to functioning as a neurotransmitter, it also acts as an inflammatory mediator [Barnes *et al* 1990; Cappugi *et al* 1992; Smith *et al* 1993] and neurohumoral regulator [Aronin *et al* 1986; Coiro *et al* 1992a+b]. Substance P is a member of the tachykinin family of peptides and acts through stimulation of the neurokinin receptors, having a particularly high affinity for the type 1 (NK_1) receptor [Stjärne *et al* 1994].

When given intra-arterially, substance P is a potent vasodilator [Löfström *et al* 1965; Eklund *et al* 1977; McEwan *et al* 1988] through an endothelium-dependent mechanism [Gross *et al* 1994] which is predominantly mediated by nitric oxide release [Cockcroft *et al* 1994]. This response is induced via stimulation of the endothelial cell NK_1 receptor [Stjärne *et al* 1994] and, increasingly, substance P is being used to assess endothelial cell function in health and disease in man [Crossman *et al* 1989; Okumura *et al* 1992; Holdright *et al* 1994; Panza *et al* 1994; Casino *et al* 1995]. Indeed, substance P has been infused into the coronary artery of man at doses up to 90 pmol/min [Crossman *et al* 1989; Okumura *et al* 1992; Holdright *et al* 1994]. However, the reproducibility and tolerability of intra-arterial substance P in the human forearm has not been fully characterised and validated. Previous studies

describing such infusions in the forearm have either used less precise methodology, including unilateral plethysmography and inclusion of the hand circulation [Eklund *et al* 1977], or have only incompletely described the nature of the response [McEwan *et al* 1988].

Therefore, the aim of the present study was to characterise the pharmacodynamics, reproducibility and tolerability of the vasodilator responses to arterial administration of substance P within the forearm vascular bed of healthy men.

3.3 METHODS

3.3.1 Subjects

Eight healthy male subjects aged between 20 and 34 years participated in a series of four studies.

3.3.2 Drugs

Synthetic pharmaceutical-grade substance P (Clinalfa AG, Läufelfingen, Switzerland) of ≥95% purity, was administered following dissolution in physiological saline (0.9% sodium chloride: Baxter Healthcare Ltd, Thetford, UK).

3.3.3 Study Design (Figure 3.1)

Subjects rested recumbent throughout each study. Strain gauges and cuffs were applied, and the brachial artery of the non-dominant arm cannulated. Saline was infused for the first 30 minutes to allow for equilibration. Forearm blood flow was measured for 3 minutes beginning at 25, 15 and 6 minutes before commencing substance P infusions. Throughout all studies, substance P was infused for 6 minutes at each dose. Forearm blood flow measurements were made for the last 3 minutes of each infusion period.

The tolerability of substance P was assessed in seven subjects who were given incremental doubling doses of substance P from 0.5 pmol/min to a maximum of 128 pmol/min [Eklund *et al* 1977], followed by a 30 minute infusion of saline (protocol a). To determine within and between day reproducibility, each subject

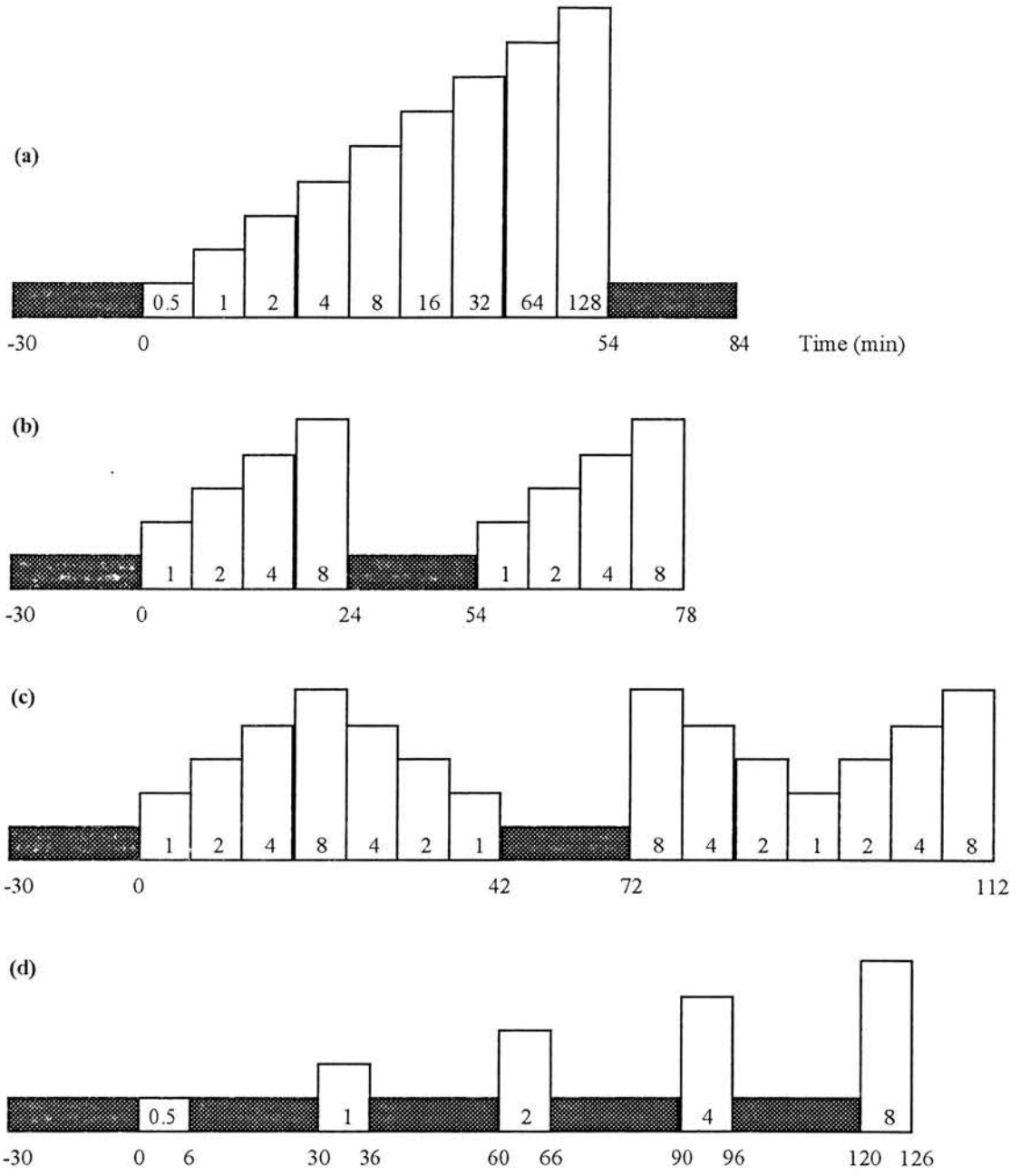


Figure 3.1
Study design: protocols a, b, c and d with saline (▩) and substance P (□) infusions.

re-attended 7 to 14 days later and received 1, 2, 4 and 8 pmol/min of substance P followed by saline for 30 minutes before receiving further incremental doses of 1, 2, 4 and 8 pmol/min of substance P (protocol b).

In order to examine whether responses to substance P undergo tachyphylaxis, seven subjects were given substance P infusions at sequential doses of 1, 2, 4, 8, 4, 2 and 1 pmol/min, followed by saline for 30 minutes before receiving substance P at sequential doses of 8, 4, 2, 1, 2, 4 and 8 pmol/min (protocol c). Seven to 14 days later, each subject re-attended and received an infusion of substance P at doses of 0.5, 1, 2, 4 and 8 pmol/min with each dose separated by a 24 minute period of saline infusion (protocol d). One subject withdrew for personal reasons after completing protocols a and b, and was replaced for protocols c and d.

3.3.4 Data Analysis And Statistics

Data were examined by two way analysis of variance (ANOVA) with repeated measures, regression analysis and two tailed Student's *t*-test using Excel v5.0 (Microsoft). All results are expressed as means ± standard errors of the mean. Statistical significance was taken at the 5% level. Within and between day reproducibility was assessed using the method of Bland & Altman [1986] and coefficients of repeatability were determined for 95% confidence intervals using the Student's *t* distribution.

3.4 RESULTS

3.4.1 Tolerability Of Intra-Arterial Substance P Infusion

Transient and patchy flushing of the infused arm occurred at all doses, whereas facial flushing was only observed at doses of ≥32 pmol/min. Five subjects received 64 pmol/min and two subjects received 128 pmol/min as the maximal dose: further infusion of substance P was discontinued because of extensive forearm skin oedema and facial flushing. There were no significant increases in heart rate or decreases in blood pressure up to 64 pmol/min (Table 3.1). No subject reported discomfort or local fullness with substance P infusion, but two subjects described transient light-headedness at doses of 16 and 64 pmol/min. In the infused forearm, patchy skin oedema developed in some subjects at 16 pmol/min and consistently, in all by 32 pmol/min. The oedema had an urticarial appearance, taking the form of a raised wheal with a yellow hue. However, there was no associated pruritis and the lesions were non-tender. The extent of oedema varied between subjects, beginning at the level of the elbow and extending distally with increasing dose. The affected areas ranged from 1 to 10 cm in diameter, but all resolved completely within 1 to 2 hours of stopping the infusion.

3.4.2 Blood Flow Responses

3.4.2.1 *Dose Range Of Vasodilator Responses*

Substance P increased blood flow in the infused forearm ($p<0.001$) in a dose-dependent manner which reached a maximum of 21 ± 3.1 mL/100mL/min (466 ± 192%) by 16 pmol/min (Table 3.2 and Figure 3.2). There was a significant increase

Table 3.1.
Systemic haemodynamics and blood flow responses to incremental doses of substance P

		Substance P Infusion (pmol/min)			
		Baseline	**2**	**16**	**64**
Blood Pressure (mmHg)	**Systolic**	140 ± 6	139 ± 4	139 ± 4	138 ± 7
	Diastolic	70 ± 4	68 ± 5	69 ± 6	65 ± 4
Heart Rate (/min)		57 ± 4	56 ± 4	61 ± 3	63 ± 3
Blood Flow (mL/100 mL/min)	**Infused Arm**	3.7 ± 0.7	11.7 ± 1.5§	20.9 ± 2.8§	21.5 ± 2.4§
	Non-infused Arm	3.4 ± 0.4	3.6 ± 0.5	4.6 ± 0.9*	8.8 ± 1.7§
% Increase in Forearm Blood Flow		-	233 ± 59 %§	466 ± 197 %§	221 ± 105 %§

*p = 0.05; §p = 0.005

Table 3.2.
Absolute forearm blood flow (mL/100 mL of tissue/min) in both arms during the four protocols

Protocol a					**Substance P Infusion** (pmol/min)					
	0	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**0**
Infused Arm	4.1 ± 0.7	7.9 ± 1.2	10.0 ± 1.5	11.7 ± 1.8	14.6 ± 2.4	16.5 ± 2.6	19.3 ± 3.1	19.7 ± 3.0	21.4 ± 2.9	5.7 ±1.2
Non-infused Arm	3.7 ± 0.4	4.0 ± 0.4	4.1 ± 0.5	3.9 ± 0.4	4.2 ± 0.6	4.3 ± 0.6	5.0 ± 0.9	6.7 ± 1.3	9.8 ± 1.6	5.7 ± 1.1
Protocol b					**Substance P Infusion** (pmol/min)					
	0	**1**	**2**	**4**	**8**	**0**	**1**	**2**	**4**	
Infused Arm	4.8 ± 0.9	9.5 ± 2.7	11.1 ± 1.8	14.4 ± 2.3	17.5 ± 2.6	5.5 ± 1.2	9.8 ± 1.8	11.0 ± 1.7	15.2 ± 2.7	18.3 ± 3.7
Non-infused Arm	4.1 ± 0.4	4.2 ± 0.5	4.1 ± 0.5	4.3 ± 0.5	4.5 ± 0.6	6.0 ± 1.1	5.9 ± 1.0	5.4 ± 0.8	5.6 ± 1.0	5.4 ± 0.8
Protocol c					**Substance P Infusion** (pmol/min)					
	0	**1**	**2**	**4**	**8**	**4**	**2**	**1**	**0**	
Infused Arm	3.4 ± 0.5	9.5 ± 0.8	11.3 ± 1.2	13.1 ± 1.4	17.5 ± 1.9	12.6 ± 1.3	10.4 ± 1.1	8.1± 0.9	4.2 ± 0.5	
Non-infused Arm	3.2 ± 0.4	3.2 ± 0.4	3.1 ± 0.5	3.0 ± 0.4	2.9 ± 0.4	3.2 ± 0.4	3.3 ± 0.4	3.2 ± 0.4	3.3 ± 0.4	
					Substance P Infusion (pmol/min)					
	0	**8**	**4**	**2**	**1**	**2**	**4**	**8**		
Infused Arm	3.7 ± 0.5	19.6 ± 1.7	12.4 ± 1.2	10.1 ± 0.9	7.3 ± 0.4	9.7 ± 0.8	11.6 ± 0.9	17.2 ± 1.7		
Non-infused Arm	3.9 ± 0.4	3.7 ± 0.6	3.3 ± 0.4	3.4 ± 0.5	3.3 ± 0.4	3.3 ± 0.3	3.5 ± 0.4	3.5 ± 0.5		
Protocol d					**Substance P Infusion** (pmol/min)					
	0	**0.5**	**0**	**1**	**0**	**2**	**0**	**4**	**0**	**8**
Infused Arm	3.4 ± 0.6	7.5 ± 0.6	3.9 ± 0.8	10.7 ± 0.9	4.3 ± 0.7	15.0 ± 1.7	4.4 ± 0.7	18.9 ± 3.1	4.4 ± 0.8	25.3 ± 3.5
Non-infused Arm	3.5 ± 0.6	3.4 ± 0.6	4.0 ± 0.7	3.9 ± 0.6	4.2 ± 0.6	4.1 ± 0.8	4.4 ± 0.7	4.6 ± 0.9	4.7 ± 0.8	4.7 ± 0.8

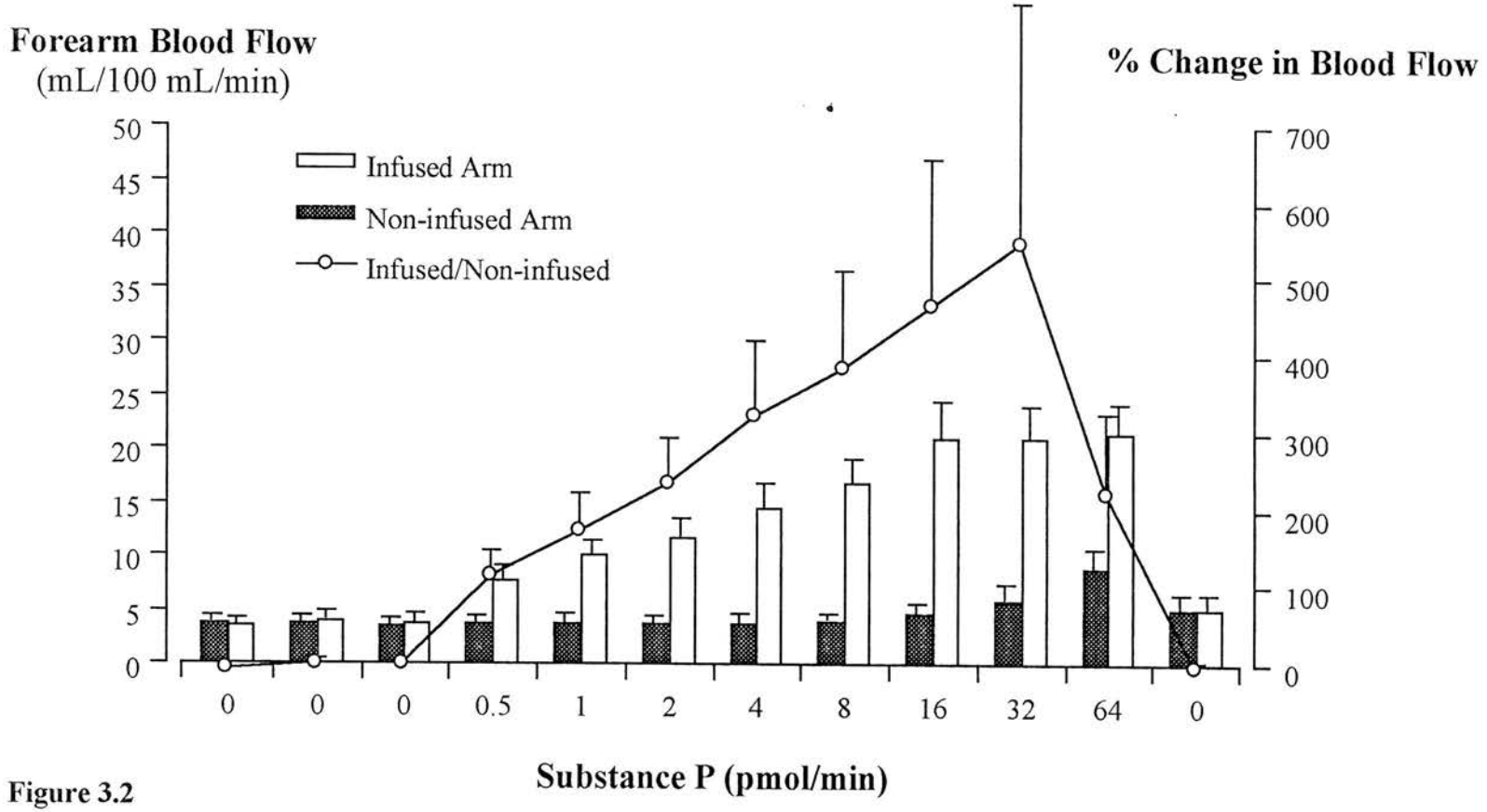


Figure 3.2
Absolute and relative forearm blood flow responses to incremental doses of substance P (protocol a).

in the blood flow of the contralateral, non-infused arm (p = 0.001, ANOVA) which was apparent from 16 pmol/min (p = 0.05, *t*-test). Relative percentage increase in blood flow of the infused to non-infused arm was dose-dependent and peaked at 32 pmol/min before declining at 64 pmol/min. The increases in blood flow had a linear relationship to the logarithm of substance P dose (Figure 3.2: $y = 174 + 242x$; $r = 0.997$, $p<0.001$) at doses $\leq$32 pmol/min.

3.4.2.2 *Reproducibility Of Vasodilator Responses*

There were no significant differences between the mean vasodilator responses to substance P either between days or within a single day (Figure 3.3A). The reproducibility of individual within day and between day responses are shown in Table 3.3. The 95% confidence intervals indicated by the coefficients of repeatability, are ~2 to 4-fold smaller for within day responses compared to those between day. These data, for a sample population of eight subjects, give 95% power to detect a mean shift in the dose response of >4-fold and >8-fold when comparing within day and between day responses respectively.

There was a trend for the magnitude of mean vasodilator responses to substance P to undergo attenuation with continuous infusions, but this did not achieve statistical significance (Figure 3.3B). Likewise, there were no significant differences between the vasodilatation to isolated discontinuous doses and incremental continuous doses of substance P (Figure 3.3C). Absolute forearm blood flows in both arms in all four protocols are shown in Table 3.2.

Table 3.3.
Within And Between Day Reproducibility For Individual Percentage Increases In Forearm Blood Flow At 1, 2, 4 and 8 pmol/min

Substance P Dose (pmol/min)	Within Day Responses			Between Day Responses		
	Mean Percentage Change in Blood Flow	Mean of the Differences	Coefficient of Repeatability	Mean Percentage Change in Blood Flow	Mean of the Differences	Coefficient of Repeatability
1	131	24.4	32	158	30.7	61
2	189	24.7	49	217	31.3	74
4	281	-35.5	34	292	57.6	144
8	362	-22.9	30	368	34	127

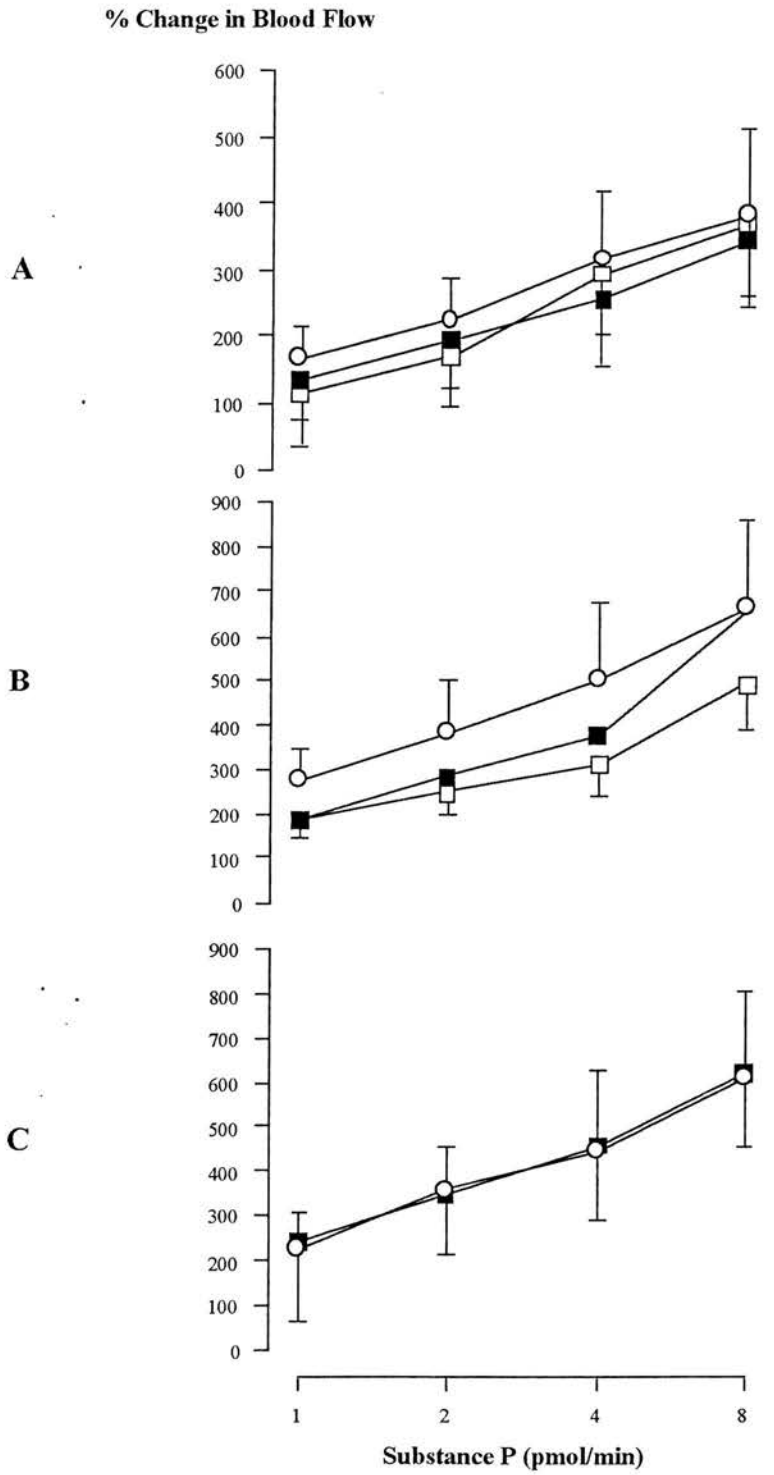


Figure 3.3

Panel A - within and between day mean increases in forearm blood flow to 1, 2, 4 and 8 pmol/min: protocol **a** dose response (○); protocol **b**, first dose response (■); and protocol **b**, second dose response (□). Panel B - development of tachyphylaxis with protocol **c**: first dose response (○); second and third dose responses (■; both were superimposable and the second response only is shown for the sake of clarity); and fourth dose response (□). Panel C - comparison of isolated discontinuous and incremental continuous infusions: protocol **c**, first dose response (■); and protocol **d**, first dose response (○).

3.5 DISCUSSION

Intra-arterial substance P infusion at doses up to and including 64 pmol/min were generally well tolerated with no significant changes in supine blood pressure and heart rate. Previous studies [Eklund *et al* 1977; Schaffalitzky *et al* 1986; Fuller *et al* 1987] using intravenous dosing have induced flushing, tachycardia and decreased blood pressure at higher doses (>150 pmol/min). However, skin oedema associated with arterial infusion has not been reported before, despite intra-brachial infusions of up to 48 pmol/min [Eklund *et al* 1977]. This disparity may reflect the previously shorter infusion times of 4 minutes per dose [Eklund *et al* 1977] or the relative purities of substance P administered. Nevertheless, our observations are consistent with the action of substance P as an inflammatory mediator inducing protein extravasation and leucocyte migration [Cappugi *et al* 1992]. These effects may affect baseline forearm circumference but not the rate of forearm expansion with plethysmographic measurements unless the rate of oedema formation were to approach that of forearm blood flow, or oedema formation were to raise tissue extracellular fluid pressure above 40 mmHg. This would be associated with substantial tissue swelling and would exceed the observed limited oedematous response.

Substance P has been used as an endothelium-dependent vasodilator in the human coronary circulation [Crossman *et al* 1989; Okumura *et al* 1992; Holdright *et al* 1994]. Total coronary blood flow is ~300 mL/min (60 to 90 mL/100 mL of tissue/min) which compares with ~50 mL/min (3 to 5 mL/100 mL of tissue/min) in

the forearm. Although left coronary artery blood flow is, therefore, ~3 to 4 times higher than that in the brachial artery, substance P does increase left coronary blood flow and at concentrations similar to those achieved in the forearm [Holdright *et al* 1994]. Therefore, given that substance P can cause oedema in the forearm and the consequences of unrecognised myocardial oedema may be serious, we would caution against the intra-coronary administration of high doses of substance P in man.

Intra-arterial substance P caused consistent and dose-dependent local increases in forearm blood flow without systemic effects at doses up to and including 8 pmol/min. In agreement with previous studies [Eklund *et al* 1977; McEwan *et al* 1988; Hirooka *et al* 1992; Cockcroft *et al* 1994; Panza *et al* 1994; Casino *et al* 1995], the vasodilator response was linearly related to the logarithm of the substance P dose. However, at doses of greater than or equal to 16 pmol/min, substance P induced systemic vasodilatation as indicated by facial flushing and an increase in forearm blood flow of the contralateral non-infused arm, although no significant decrease in blood pressure, or increase in heart rate, was observed. The use of comparative increases in forearm blood flow of the infused forearm with respect to the non-infused arm, becomes invalid at systemic doses and accounts for the breakdown of the log-linearity of responses at doses of more than 32 pmol/min.

McEwan and colleagues [McEwan *et al* 1988] have shown that substance P at 1 pmol/min undergoes tachyphylaxis when infused continuously for 10 minutes or more. However, we did not find a statistically significant attenuation of the responses when administering decremental doses of substance P. Moreover, we found no

greater response with incremental discontinuous infusions than with incremental continuous doses of substance P. Mean increases in forearm blood flow to substance P at doses of 1 to 8 pmol/min were of equivalent magnitude both within day and between days. Eklund and colleagues [Eklund *et al* 1977] have reported variable measurements with substance P, but their results are confounded by utilising cruder methodology with unilateral forearm plethysmography and inclusion of the hand circulation. In contrast to the muscular forearm, the hand is predominantly skin and has a heterogeneous circulation which is regulated in a complex and non-linear manner [Webb 1995]. We have demonstrated good reproducibility when comparing individual within day responses. Based on a sample size of eight, the confidence intervals would indicate that we would have 95% power to detect >4-fold shift in the dose-response relationship when comparing within day responses at each dose.

In summary, we have found using within day comparisons, that at doses up to 8 pmol/min, vasodilatation to substance P is generally well tolerated and highly reproducible. Such methodology should provide a practical and sensitive method of assessing the *in vivo* efficacy of peripheral NK_1 receptor antagonism in man [Beattie *et al* 1994].

CHAPTER 4

SUBSTANCE P INDUCED VASODILATATION IS MEDIATED BY THE NEUROKININ TYPE 1 RECEPTOR BUT DOES NOT CONTRIBUTE TO BASAL VASCULAR TONE IN MAN

Newby DE, Sciberras DG, Ferro CJ, Gertz BJ, Sommerville D, Majmadar A, Lowry RC, Webb DJ.
Substance P induced vasodilatation is mediated by the neurokinin type 1 receptor but does not contribute to basal vascular tone in man.
Br J Clin Pharmacol 1999;**48**:336-344.

4.1 SUMMARY

Following intravenous administration of its prodrug, L-758,298, we assessed the pharmacodynamics of L-754,030, a novel and highly selective NK_1 receptor antagonist, by examining systemic haemodynamics and the blood flow responses to intra-arterial substance P infusion. Sixteen healthy male volunteers participated in a double blind, randomised, placebo controlled crossover trial of L-758,298. Forearm blood flow was measured using venous occlusion plethysmography during intra-brachial substance P infusion (0.125-128 pmol/min). In Part 1, eight subjects received substance P infusions before and during placebo, 0.25 mg, 1 mg or 5 mg of L-758,298. In Part 2, eight subjects received substance P infusions 24 hours after placebo or 1.43 mg of L-758,298. L-758,298 caused dose-dependent inhibition of substance P induced vasodilatation during administration ($p<0.001$). Placebo adjusted differences (95% CI) in baseline forearm blood flow, mean arterial pressure and heart rate showed no relevant changes with 5 mg of L-758,298 (>1400–fold shift in substance P response): 0.00 (-0.49 to +0.49) mL/100 mL/min, 1.0 (-3.2 to +5.2) mmHg and 1.9 (-5.9 to +9.7) /min respectively. Twenty-four hours after 1.43 mg of L-758,298, there was ~34–fold shift in response to substance P induced vasodilatation ($p<0.008$) at plasma L-754,030 concentrations of 2 to 3 ng/mL. L-758,298 was generally well tolerated without serious adverse events. Substance P induced forearm vasodilatation is mediated by the endothelial cell NK_1 receptor in man but endogenous substance P does not appear to contribute to the maintenance of peripheral vascular tone or systemic blood pressure.

4.2 INTRODUCTION

Substance P is a widely distributed endecapeptide which is found principally in the neural tissue of the central, peripheral and enteric nervous systems [Hökfelt *et al* 1977; Lundberg *et al* 1979; Pernow 1983; Aronin *et al* 1986]. The physiological functions of substance P include neurotransmission in primary sensory neurones with particular involvement in nociception and emesis. In addition to functioning as a neurotransmitter, it also acts as an inflammatory mediator [Barnes *et al* 1990; Cappugi *et al* 1992; Smith *et al* 1993] and neurohumoral regulator [Hökfelt *et al* 1977; Coiro *et al* 1992a+b].

Substance P is a member of the tachykinin family of peptides and acts through stimulation of the neurokinin receptors, having a particularly high affinity for the type 1 (NK_1) receptor [Stjärne *et al* 1994]. When given intra-arterially, substance P is a potent vasodilator [Löfström *et al* 1965; Eklund *et al* 1977; McEwan *et al* 1988] through an endothelium-dependent mechanism [Gross *et al* 1994] which is partly mediated by nitric oxide release [Cockcroft *et al* 1994]. In animal studies, this response is induced via stimulation of the endothelial cell NK_1 receptor [Stjärne *et al* 1994] although, to date, this has not been confirmed *in vivo* in man. Substance P is found in perivascular neural tissue [Weihe *et al* 1981] and has been postulated to play a role in the regulation of vascular tone [Crossman *et al* 1989; Quyyumi *et al* 1997].

Antagonism of the NK_1 receptor has potentially diverse therapeutic indications such as in the treatment of pain, inflammation and emesis [Navari *et al* 1999]. L-754,030 (2-(R)-(1-(R) -3,5-bis (trifluoromethyl) phenylethoxy)-3-(S)-(4-fluoro) phenyl-4-(3-(5-oxo-4H-1,2,4-triazolo)methyl morpholine; also known as MK-869) is a long acting, highly selective, competitive NK_1 receptor antagonist with poor solubility in aqueous solution. L-754,030 is more selective for the NK_1 than the NK_3 (3,000–fold) or the NK_2 and other G-protein linked receptors and ion channels (>50,000–fold) [Kramer *et al* 1998]. N-phosphorylation of L-754,030 produces L-758,298, a prodrug which is readily soluble in aqueous solutions. L-758,298 undergoes rapid *in vivo* conversion to L-754,030 and thereby provides a prodrug which can be administered intravenously.

The primary aims of the present study were: first, to determine the ability of L-754,030 to inhibit substance P induced vasodilatation during and 24 hours after intravenous administration of L-758,298; second, to confirm that substance P induced vasodilatation is mediated via the endothelial cell NK_1 receptor in man; and third, to determine whether endogenous substance P regulates peripheral vascular tone or blood pressure in man. An additional important aim of the study was to evaluate the safety and tolerability of single intravenous doses of L-758,298 in healthy male volunteers.

4.3 METHODS

4.3.1 Subjects

Healthy non-smoking men aged between 18 and 45 years participated in a series of studies which were undertaken with the approval of the Lothian Research Ethics Committee and the written informed consent of each subject.

4.3.2 Drug Administration

Synthetic pharmaceutical-grade substance P (Clinalfa AG, Läufelfingen, Switzerland) of ≥95% purity, was administered following dissolution in saline.

Matched placebo and L-758,298 (Merck Research Laboratories, West Point, USA) were reconstituted with 0.9% saline in glass vials containing 50 mg of mannitol alone or 6 mg of L-758,298 and 50 mg of mannitol respectively. The 5 mg initial dose of L-758,298 was chosen to exceed the ID_{90} of 50 μg/kg in the guinea pig sensorotoxin-induced systemic vascular leak model (Merck Research Laboratories, Terlings Park, UK). Cannulae were inserted into the veins of the antecubital fossae of both arms. L-758,298 was administered into the dominant arm via a 19-G cannula (Wallace Y-Can; Wallace Ltd, Colchester, UK) and venous samples were withdrawn from the non-dominant arm via a 17-G cannula (Venflon; BOC Ohmeda AB, Helsingborg, Sweden).

4.3.3 Pharmacokinetics, Safety and Tolerability

Ten mL of blood was withdrawn from the non-dominant arm before and after the incremental infusion of substance P and admixed with 1 mL of 1% disodium EDTA. Blood samples were placed on ice before being centrifuged at 2,000 *g* for 30 minutes. Plasma was frozen and stored at -80°C prior to assay. Plasma L-754,030 concentrations were determined using high performance liquid chromatography and mass spectrometry using an internal standard (L-752,611). The assay was validated over a concentration range of 1 to 500 ng/mL with a limit of detection at 1 ng/mL and a coefficient of variation of <9%.

Safety and tolerability assessments were made before, during and following completion of the study and included: clinical examination, repeated questioning for symptoms, clinical chemistry screen (liver enzymes, bilirubin, electrolytes, urea, creatinine, protein and albumin), haematology screen (full blood and differential count), urinalysis and 12-lead electrocardiogram.

4.3.4 Study Design (Figure 4.1)

Subjects attended at 9.00 am, rested recumbent throughout each study and intravenous cannulae were inserted into each arm. Strain gauges and cuffs were applied, and the brachial artery of the non-dominant arm cannulated. Saline was infused into the arterial cannula for the first 30 minutes to allow for equilibration. Forearm blood flow was measured for 3 minutes beginning at 23, 13 and 3 minutes before commencing substance P infusions. Throughout all studies, substance P was

infused for 10 minutes at each dose. Forearm blood flow measurements were made from 3 to 6 minutes of each infusion period.

4.3.4.1 *Screening*

Before inclusion in the main study, subjects received intra-arterial infusions of substance P at 0.125, 0.5, 2, 8 and 32 pmol/min [Newby *et al* 1997a]. To reduce overall variability in the responses, subjects were recruited to the main study if 2 pmol/min of substance P increased forearm blood flow by ≥100% and ≤500%.

4.3.4.2 *Part 1 – Maintenance L-758,298 Infusion*

On each occasion, eight subjects received incremental intra-arterial infusions of substance P at 0.125, 0.5, 2 and 8 pmol/min followed by 60 minutes of saline. A second infusion of substance P was then administered at 0.125, 0.5, 2, 8, 32 and 128 pmol/min [Newby *et al* 1997a].

At the beginning of the intervening 60 minute saline infusion, subjects received a double blind, randomised intravenous infusion of either L-758,298 or placebo: Table 4.1. An intravenous bolus (two-thirds of the total dose over 20 minutes) was given followed by a continuous maintenance infusion (one-third of the total dose over the subsequent 100 minutes) throughout the second challenge of intra-arterial substance P administration. From earlier phase I pharmacokinetic studies in man (Merck; data on file), this dosage regimen was predicted to produce a stable plasma concentration of L-754,030 during the second incremental infusion of intra-arterial substance P. Plasma samples to measure L-754,030 concentrations were taken 60 and

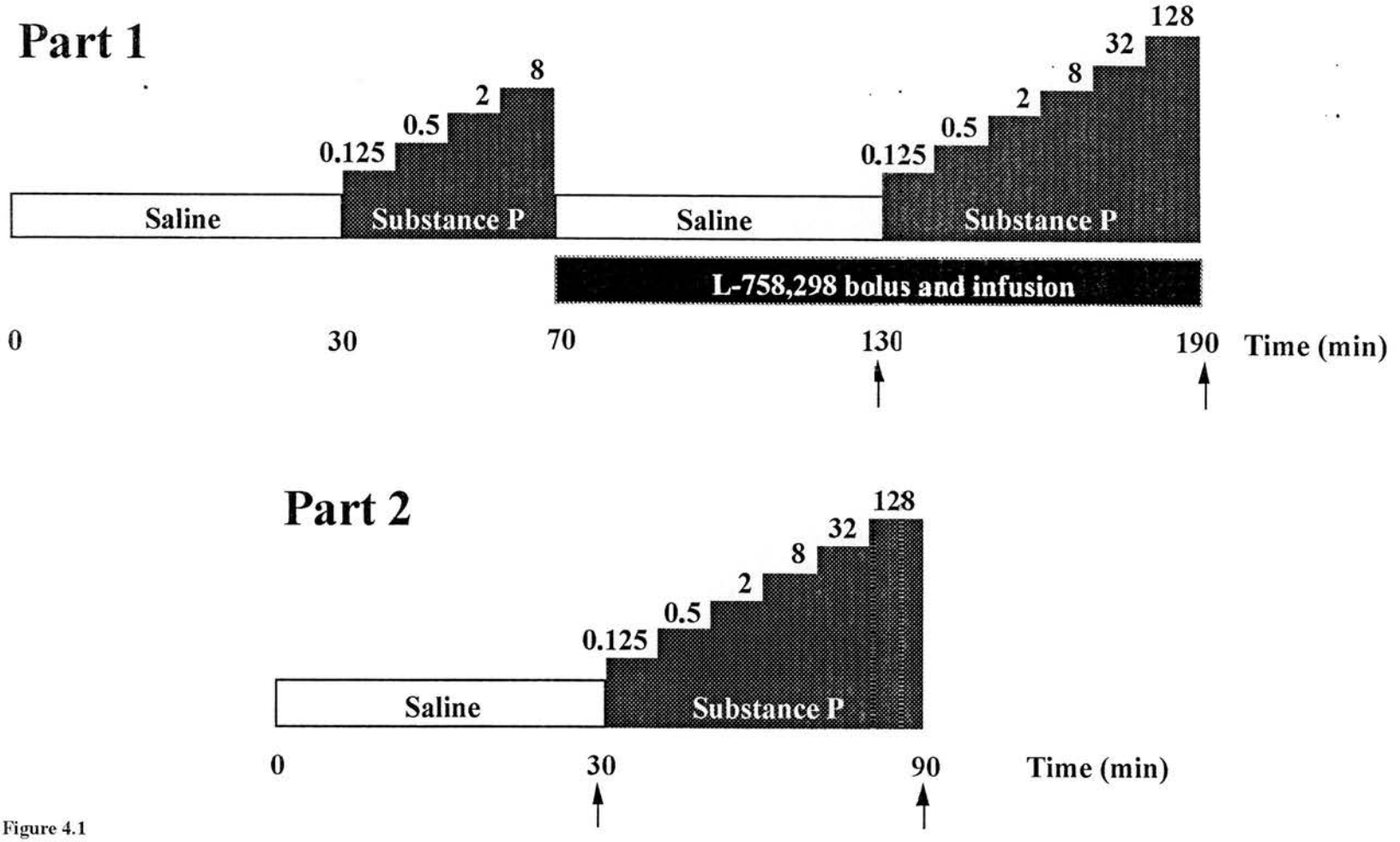


Figure 4.1
Schematic of protocol.
Doses of substance P given in pmol/min. Arrows indicate blood sampling time points for the estimation of plasma L-754,030 concentrations. Part 2 infusions were commenced 24 hours after 1.43 mg of intravenous L-758,298 administration.

Table 4.1.
Schematic of drug allocation

Subject	Week 1	Week 2*	Week 3†	Week 4
1	Placebo	D_1	D_2	D_3
2	D_1	Placebo	D_2	D_3
3	D_1	D_2	Placebo	D_3
4	D_1	D_2	D_3	Placebo
5	Placebo	D_1	D_2	D_3
6	D_1	Placebo	D_2	D_3
7	D_1	D_2	Placebo	D_3
8	D_1	D_2	D_3	Placebo

D_1 = 5 mg of L-758,298
*If the PD_{100} increases by >40-fold then D_2 = 1 mg of L-758,298; if <40-fold, D_2 = 20 mg
†If the PD_{100} continues to be >40-fold then D_3 = 0.25 mg of L-758,298 or <40-fold, D_3 = 80 mg: otherwise the study is completed after 3 weeks i.e. D_3 = placebo

120 minutes after the start of the intravenous infusion of L-758,298 or placebo. In the first week, subjects received a total dose of 5 mg of L-758,298 or placebo. The next dose administered in the second week would be increased to 20 mg if the PD_{100} (defined below) was increased by <40-fold by 5 mg of L-758,298 or reduced to 1 mg if >40-fold (based on data from six subjects). The same criteria were applied to determine whether the final dose (80 mg or 0.25 mg) of L-758,298 was to be administered, or the study completed after two doses of L-758,298 and placebo (based on data from four subjects; see Table 4.1). If the third dose was not to be given then this would be replaced by placebo and the study terminated after 3 weeks. To maintain double blind randomisation, all data analysis and dosage decisions were made at the central co-ordinating centre (Merck Research Laboratories, Terlings Park, UK) independent of the investigators.

4.3.4.3 *Part 2 – 24 Hours Post L-758,298 Infusion*

Eight further subjects attended on two occasions, one week apart, and received an intravenous infusion of either L-758,298 or placebo over 30 minutes in a double blind randomised manner. Subjects returned 24 hours later to receive infusions of intra-arterial substance P at 0.125, 0.5, 2, 8, 32 and 128 pmol/min [Newby *et al* 1997a]. Plasma samples to measure L-754,030 concentrations were taken 24 and 25 hours after the start of the intravenous infusion of L-758,298 or placebo. The dose of L-758,298 was derived from the pharmacodynamic and pharmacokinetic data of part 1 and was chosen to produce an approximately 40–fold increase in the PD_{100} 24 hours after L-758,298 administration. L-754,030 has a plasma half life of 15 ± 4 hours in man.

4.3.5 Data Analysis And Statistics

Percentage change in forearm blood flow from baseline was calculated using the ratios of flows in the two arms for each of the substance P infusions. Natural logarithm transformations of the substance P doses were used to estimate individual PD_{100} values using linear regression techniques. The PD_{100} was defined as the interpolated or extrapolated dose of substance P which provokes a 100% increase in forearm blood flow. To determine whether there was a within day difference in PD_{100} following placebo, an analysis of variance (ANOVA) with the terms 'subjects' and 'time' (predose and postdose) was used to analyse the natural logarithm transformed PD_{100} data from the placebo treatment group in Part 1.

The influence of each dose of L-758,298 in Parts 1 and 2 on the forearm blood flow response to substance P were evaluated relative to placebo. In these analyses, the PD_{100} after administration of a placebo dose was used as the control for assessment of the fold shifts in PD_{100} due to the administration of the various doses of L-758,298. An ANOVA with the terms 'subject' and 'treatment' was used to analyse the natural logarithm transformed PD_{100} data from the treatments in Part 1. An ANOVA appropriate for a two period crossover study was used to analyse the natural logarithm transformed PD_{100} data from the treatments in Part 2. These ANOVA analyses were used to estimate the geometric means, their ratios and 95% confidence intervals for the geometric mean ratio for PD_{100}.

Mean arterial pressure, heart rate and blood flow data were examined, where appropriate, by multifactorial ANOVA with repeated measures and paired Student's *t*-test. All results are expressed as mean ± standard error of the mean. Statistical significance was taken at the 5% level.

4.4 RESULTS

Twenty-two healthy male volunteers were required to identify 16 subjects who met the entry criteria: forearm blood flow increased by >500% in two subjects and <100% in four subjects. The sixteen volunteers subsequently recruited were aged 30 ± 2 years (range 20 to 40 years) and weighed 76 ± 3 kg (range 61 to 95 kg).

There were no significant baseline differences in forearm blood flow, blood pressure or heart rate between any parts of the study.

4.4.1 Part 1

Substance P caused dose-dependent vasodilatation in the non-dominant arm ($p<0.001$; ANOVA) during the first incremental infusion of substance P which was not significantly different between screening or the four separate study days.

L-758,298 was administered in three descending doses: 5, 1 and 0.25 mg. During placebo and L-758,298 infusion, there were no significant changes in blood pressure, heart rate, or blood flow in the dominant forearm (Table 4.2). Placebo adjusted differences (95% CI) in dominant forearm blood flow, mean arterial pressure and heart rate with 5 mg of L-758,298 were 0.00 (-0.49 to 0.49) mL/100 mL/min, 1.0 (-3.2 to 5.2) mmHg and 1.9 (-5.9 to 9.7) /min respectively. Plasma concentrations of L-754,030 were not significantly different immediately before and after the second incremental infusion of substance P (Table 4.2).

Table 4.2.
Mean arterial pressure (MAP), heart rate (HR), forearm blood flow and plasma L-754,030 concentrations during substance P challenges in parts 1 and 2 (See Figure 1) (Mean ± SEM)

Study Protocol	Baseline					Maximum Substance P Dose§				
	MAP (mmHg)	HR (/min)	Non-dominant Arm Blood Flow (mL/100 mL/min)	Dominant Arm Blood Flow (mL/100 mL/min)	Plasma L-754,030 Concentration (ng/mL)	MAP (mmHg)	HR (/min)	Non-dominant Arm Blood Flow (mL/100 mL/min)	Dominant Arm Blood Flow (mL/100 mL/min)	Plasma L-754,030 Concentration‡ (ng/mL)
Part 1										
First Substance P Challenge										
Placebo	90 ± 2	55 ± 3	2.9 ± 0.2	2.9 ± 0.4	-	88 ± 2	60 ± 4	12.3 ± 1.5*	2.9 ± 0.3	-
5 mg	85 ± 2	57 ± 2	3.5 ± 0.4	3.0 ± 0.5	-	89 ± 2	59 ± 2	16.6 ± 2.9*	3.0 ± 0.5	-
1 mg	87 ± 3	51 ± 1	3.2 ± 0.4	2.8 ± 0.4	-	91 ± 3	54 ± 2	9.7 ± 2.2*	2.8 ± 0.4	-
0.25 mg	90 ± 3	60 ± 2	3.0 ± 0.3	2.7 ± 0.3	-	91 ± 3	62 ± 4	12.1 ± 1.4*	2.7 ± 0.3	-
Second Substance P Challenge										
Placebo	95 ± 2	57 ± 4	3.4 ± 0.3	3.1 ± 0.3	-	93 ± 3	59 ± 3	20.1 ± 3.1*	5.8 ± 0.8†	-
5 mg	88 ± 2	57 ± 3	3.6 ± 0.5	3.2 ± 0.4	75.4 ± 0.4	90 ± 2	57 ± 3	5.1 ± 0.6†	3.5 ± 0.4	86.1 ± 10.7
1 mg	90 ± 3	54 ± 2	3.3 ± 0.4	3.0 ± 0.3	10.0 ± 1.1	92 ± 3	58 ± 3	6.0 ± 0.9*	3.0 ± 0.4	11.1 ± 1.0
0.25 mg	92 ± 2	56 ± 4	2.7 ± 0.2	2.8 ± 0.3	2.6 ± 0.2	93 ± 3	64 ± 4	7.8 ± 1.2*	2.8 ± 0.3	2.8 ± 0.2
Part 2										
Placebo	86 ± 2	55 ± 3	3.3 ± 0.4	2.7 ± 0.4	-	88 ± 2	61 ± 5	22.7 ± 2.0*	7.3 ± 0.8*	-
1.43 mg	87 ± 4	55 ± 2	3.2 ± 0.4	2.6 ± 0.3	2.3 ± 0.3	86 ± 3	57 ± 2	11.9 ± 1.8*	3.5 ± 0.53†	2.2 ± 0.3

*p<0.001 (*vs* baseline; *t*-test); †p<0.05 (*vs* baseline; *t*-test).
‡n=8 for 0.25 mg, n=7 for 1 mg, n=8 for 1.43 mg and n=2 for 5 mg of L-758,298.
§Maximum substance P dose: 8 pmol/min for Part 1, pre-infusion; 128 pmol/min for part 1, during infusion; 128 pmol/min for Part 2

In comparison to the response to the first incremental infusion, the response to the second infusion of substance P was significantly different following placebo ($p<0.006$) and all doses of L-758,298 in a dose-dependent manner (Table 4.2; Figure 4.2). The geometric mean PD_{100} (95% CI) increased by 1.85-fold (1.27 to 2.86) during placebo infusion. The influence of each dose of L-758,298 on the forearm blood flow response to substance P was examined relative to placebo. The geometric mean PD_{100} (95% CI) increased by 30–fold (9-99) with 0.25 mg, 319–fold (98 to 1044) with 1 mg and >1400–fold with 5 mg of L-758,298 ($p<0.001$ for all).

Forearm blood flow in the dominant arm increased at doses ≥32 pmol/min of substance P only during placebo ($p=0.02$ *vs* baseline; paired *t*-test). Heart rate and blood pressure did not change significantly (Table 4.2).

4.4.2 Part 2

Following placebo infusion, substance P again caused dose-dependent vasodilatation in the non-dominant arm ($p<0.001$; ANOVA: Figure 4.3). However, 24 hours following intravenous administration of 1.43 mg of L-758,298, substance P induced vasodilatation was significantly inhibited (Table 4.2; Figure 4.3). The geometric mean PD_{100} (95% CI) was increased by 34–fold (4 to 299; $p<0.008$). The PD_{100} and plasma L-754,030 concentrations (Table 4.2) were similar to those obtained with 0.25 mg of L-758,298 in Part 1.

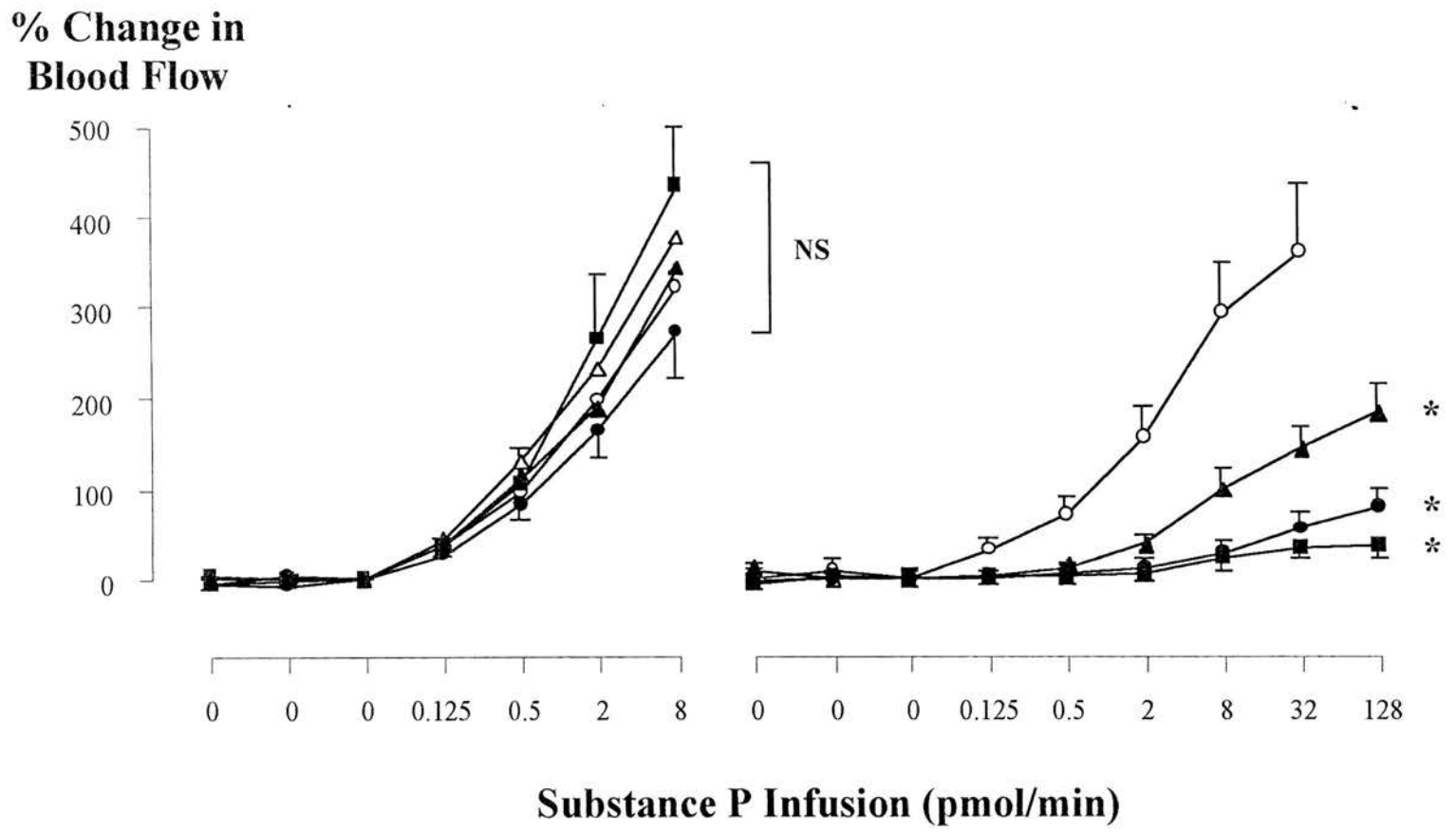


Figure 4.2

Part 1: Forearm blood flow responses to intra-brachial substance P infusion before (left panel) and during placebo or L-758,298 infusion (right panel). Mean ± SEM.

o Placebo; ▲ 0.25 mg, ● 1 mg and ■ 5 mg of L-758,298

* $p<0.001$; L-758,298 *vs* placebo, ANOVA.

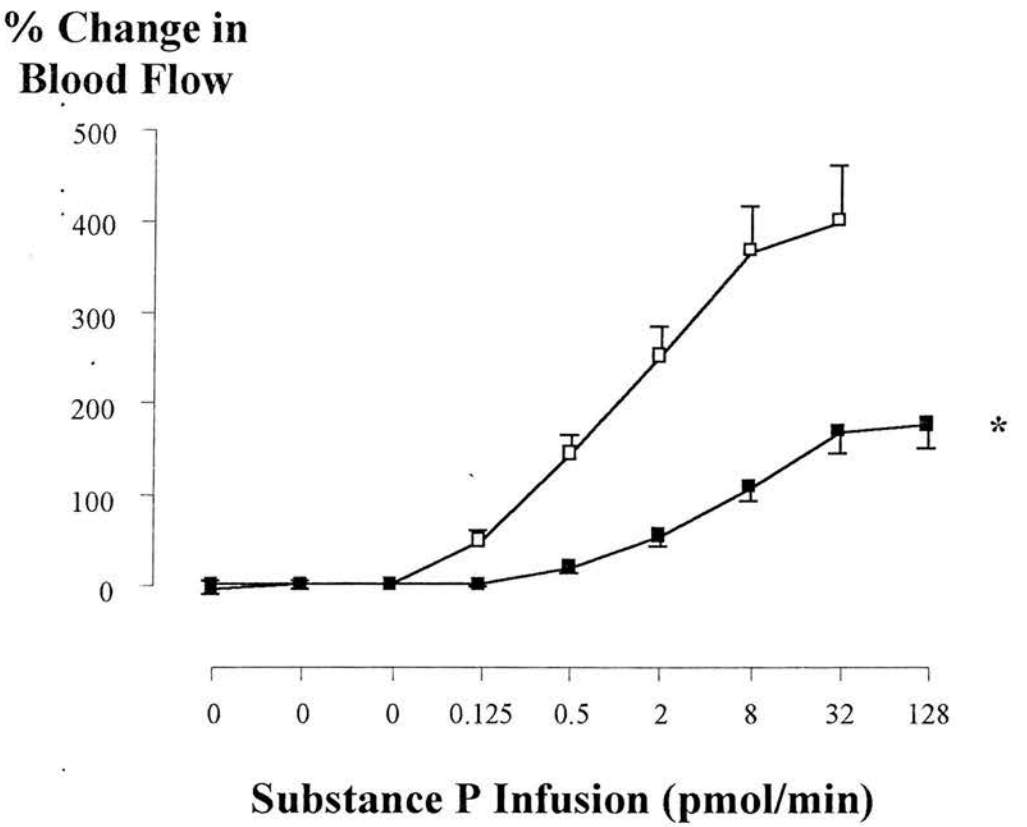


Figure 4.3
Part 2: Forearm blood flow responses to intra-brachial substance P infusion. Mean ± SEM.
□ Placebo; ■ 1.43 mg of L-758,298
* $p<0.001$; L-758,298 *vs* placebo, ANOVA.

Forearm blood flow in the dominant arm increased at doses ≥32 pmol/min following placebo ($p<0.02$ *vs* baseline; paired *t*-test) and at 128 pmol/min following 1.43 mg of L-758,298 ($p=0.03$ *vs* baseline; paired *t*-test). Heart rate and blood pressure did not change significantly (Table 4.2).

4.4.3 Concentration-Response Relationship

Plasma L-754,030 concentrations significantly correlated with the logarithm of the PD_{100} of the rate of substance P infusion ($r=0.62$, $p=0.003$; Figure 4.4).

4.4.4 Safety And Tolerability

L-758,298 was generally well tolerated by all the subjects, with no excess of adverse events (mild headaches and back pain) in comparison to placebo. There were no serious adverse events during the study and no clinically significant abnormalities detected on safety monitoring (urinalysis, haematology, clinical chemistry and electrocardiography).

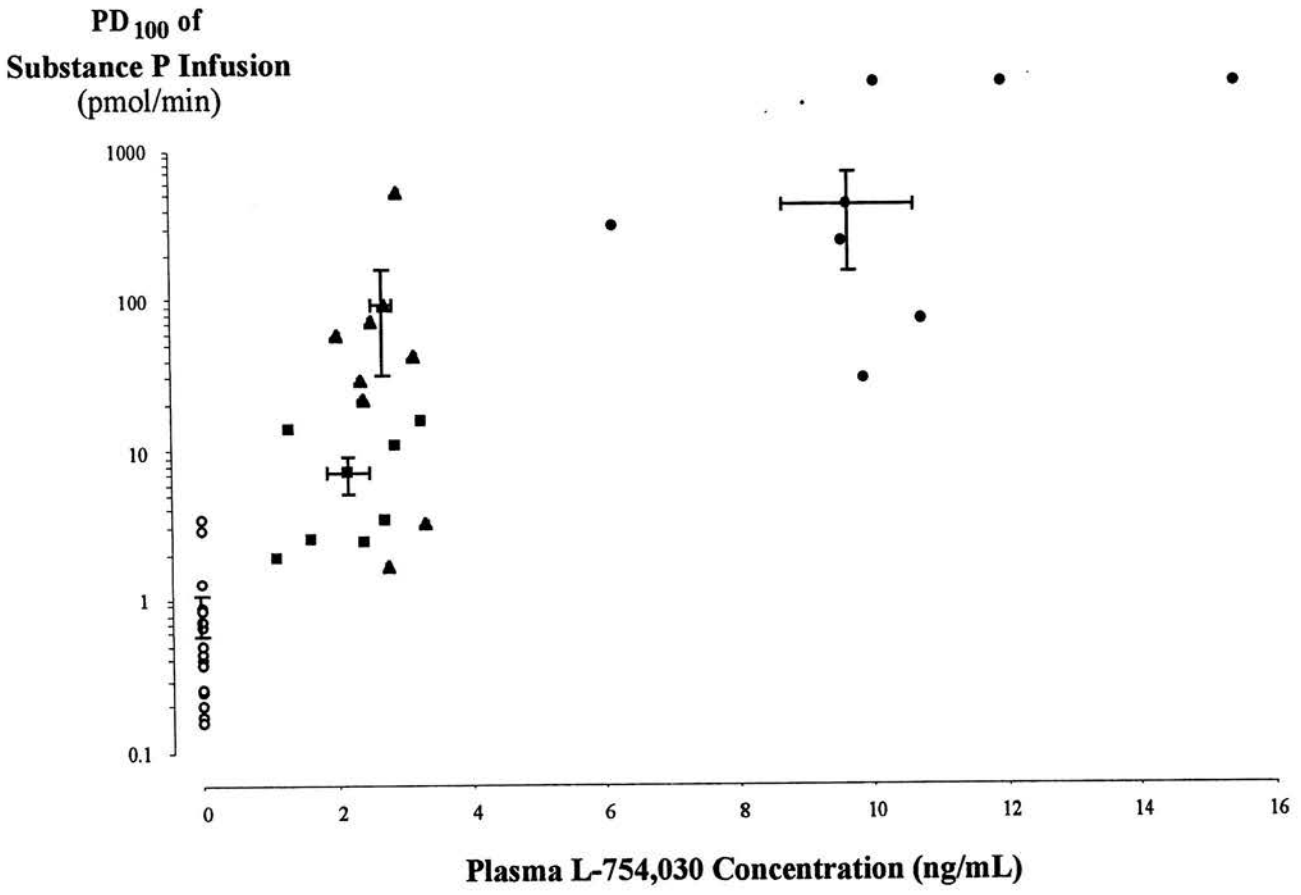


Figure 4.4
Concentration-response relationship between the plasma concentration of L-754,030 and the PD_{100} values for the rate of substance P infusion. Individual values shown with mean ± SEM. Three points with indeterminate PD_{100} values >1454 are not shown.
° Placebo; ■ 0.25 mg, ▲ 1 mg and ● 24 hours after 1.43 mg of L-758,298
r=0.62, p=0.003

4.5 DISCUSSION

For the first time, we have shown that substance P induced forearm vasodilatation is inhibited by a selective NK_1 receptor antagonist *in vivo* in man. During L-758,298 infusion, substance P induced forearm vasodilatation was inhibited in a dose-dependent manner. At the highest dose of L-758,298, the vasodilator response to substance P was abolished at doses up to 8 pmol/min, suggesting that substance P mediated vasodilatation is entirely dependent on the endothelial NK_1 receptor. Moreover, persistent inhibition of substance P induced vasodilatation was present 24 hours after L-758,298 infusion.

Despite previous proposals [Crossman *et al* 1989, Quyyumi *et al* 1997], it would appear that substance P, acting via the NK_1 receptor, does not play a role in the regulation of peripheral vascular tone or blood pressure. We observed no alterations in baseline forearm blood flow or systemic haemodynamics following L-758,298 infusion despite a greater than 1454-fold shift in the PD_{100} for substance P induced forearm vasodilatation. The 95% confidence intervals indicate that if substance P provides any contribution to basal peripheral vascular tone or systemic haemodynamics then it is rather small.

We have previously shown that repeated responses to substance P are reproducible and well tolerated [Newby *et al* 1997a]. In the current study, we have again seen good reproducibility of both within-day and between-day responses to intra-arterial substance P infusions. Systemic effects, such as increases in contralateral forearm

blood flow, were also observed at substance P doses of ≥32 pmol/min without significant changes in heart rate or blood pressure. Moreover, these increases in contralateral forearm blood flow were also inhibited by L-758,298 infusion. Finally, L-758,298 infusion was generally well tolerated without any significant adverse events.

4.5.1 Study Limitations

Because L-758,298 was administered systemically, there remains the possibility that compensatory mechanisms may have obscured a potential haemodynamic effect. Direct intra-arterial administration of an NK_1 receptor antagonist would provide a more precise method of assessing the role of substance P in the regulation of vascular tone. However, L-758,298 is a prodrug which requires conversion by systemic hepatic phosphatases to the active form L-754,030 and its intra-arterial administration would, therefore, not result in local NK_1 receptor antagonism.

In the present study, we did not assess the selectivity of L-754,030 for the NK_1 receptor by comparing substance P induced vasodilatation with an alternative non-NK_1 receptor mediated, endothelium-dependent vasodilator, such as bradykinin or acetylcholine, and this requires confirmation in future studies. However, L-754,030 has been shown to be highly selective for the NK_1 receptor [Kramer *et al* 1998].

We conclude that substance P induced forearm vasodilatation is mediated by the endothelial cell NK_1 receptor in man. Endogenous substance P does not appear to contribute to the maintenance of peripheral vascular tone or systemic blood pressure.

In this study, intravenous L-758,298 was generally well tolerated with L-754,030 causing long lasting and potent NK_1 receptor antagonism in man.

CHAPTER 5

AN *IN VIVO* MODEL FOR THE ASSESSMENT OF ACUTE FIBRINOLYTIC CAPACITY OF THE ENDOTHELIUM

Newby DE, Wright RA, Ludlam CA, Fox KAA, Boon NA, Webb DJ.
An *in vivo* model for the assessment of
the acute fibrinolytic capacity of the endothelium.
Thromb Haemost 1997;**78**:1242-1248.

5.1 SUMMARY

The effects on blood flow and plasma fibrinolytic and coagulation parameters of intra-arterial substance P, an endothelium-dependent vasodilator, and sodium nitroprusside, a control endothelium-independent vasodilator, were studied in the human forearm circulation. At subsystemic locally active doses, both substance P (2 to 8 pmol/min) and sodium nitroprusside (2 to 8 μg/min) caused dose-dependent vasodilatation ($p<0.001$ for both) without affecting plasma concentrations of PAI-1, von Willebrand factor antigen or factor VIII:C activity. Substance P caused local increases in t-PA antigen and activity ($p<0.001$) in the infused arm while sodium nitroprusside did not. At higher doses, substance P increased blood flow and t-PA concentrations in the non-infused arm. We conclude that brief, locally active and subsystemic infusions of intra-arterial substance P cause a rapid and substantial local release of t-PA which appear to act via a flow and nitric oxide independent mechanism. This model should provide a useful and selective method of assessing the *in vivo* capacity of the forearm endothelium to release t-PA acutely.

5.2 INTRODUCTION

Endothelial cells of the pre-capillary arterioles and post-capillary venules [Levin *et al* 1994] synthesise and constitutively secrete tissue-type plasminogen activator (t-PA) and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1). The release of t-PA may be rapidly increased through the translocation of a dynamic intracellular storage pool [van den Eijnden-Schrauwen *et al* 1995] in response to stimulation by blood coagulation and humoral factors [Emeis 1992]. Acute t-PA release plays a pivotal role in endogenous fibrinolysis and this is exemplified by a plasminogen activator deficient gene knockout mouse model that exhibits an increased incidence of endotoxin-induced thrombosis [Carmeliet *et al* 1994]. The time course of t-PA release is important, with thrombus dissolution being much more effective if t-PA is incorporated during, rather than after, thrombus formation [Brommer 1984; Fox *et al* 1984]. Thus, the speed with which, and extent to which, t-PA can be released from endothelial cells may have a substantial impact on the efficacy of endogenous fibrinolysis.

When studying *in vivo* vascular responses in man, systemic drug administration can cause concomitant effects on other organ systems, such as the liver, brain, kidney and heart, as well as influence neurohumoral reflexes through changes in systemic haemodynamics. Therefore, because of these confounding influences, vascular and humoral responses cannot be wholly attributed to a direct effect of the drug on the blood vessels. Endogenous fibrinolysis in man has been assessed using systemic infusion of agents such as desmopressin [Ludlam *et al* 1980; Duprez *et al* 1991] and

angiotensin II [Ridker *et al* 1993b]. These agents are vasoactive, producing changes in blood pressure and regional blood flow, as well as having widespread effects on many tissues. Thus, changes in systemic fibrinolytic parameters might be attributable to a number of factors including changes in hepatic release and clearance of t-PA and PAI-1, and the concomitant release of other stimulatory, vasoactive and humoral mediators. In contrast, the use of bilateral forearm blood flow measurements coupled with unilateral brachial artery infusion of vasoactive drugs at subsystemic, locally active doses, provides a powerful and reproducible method of directly assessing vascular responses *in vivo* [Benjamin *et al* 1995; Webb 1995]. Combined with bilateral forearm venous sampling, this technique permits the assessment of local release of tissue and endothelium-derived factors [Plumpton *et al* 1995].

The initial aim of the present study was to assess the acute release of coagulation and fibrinolytic factors within the forearm vascular bed in response to intra-arterial substance P using an ascending dose design to define the dose at which systemic effects intervene. Thereafter, endothelial cell release of these factors was assessed in response to locally active doses of substance P and a control endothelium-independent nitric oxide donor, sodium nitroprusside [Panza *et al* 1994; Casino *et al* 1995].

5.3 METHODS

5.3.1 Subjects

Sixteen healthy non-smoking men aged between 20 and 34 years participated in two studies which were undertaken with the approval of the local Research Ethics Committee and in accordance with the Declaration of Helsinki.

5.3.2 Drugs

Pharmaceutical-grade substance P (Clinalfa AG, Läufelfingen, Switzerland) and sodium nitroprusside (Nipride; Roche, Welwyn Garden City, UK) were administered following dissolution in saline (0.9%: Baxter Healthcare Ltd, Thetford, UK).

5.3.3 Study Design

Subjects rested recumbent throughout each study. Strain gauges and cuffs were applied and the brachial artery of the non-dominant arm cannulated. Measurements of forearm blood flow were made between 3 and 6 minutes of each infusion period unless otherwise stated. Before participating in one of the following protocols, saline was infused for the first 30 minutes to allow time for equilibration, with forearm blood flow measured every 10 minutes and the final measurement taken as basal blood flow.

5.3.3.1 *Dose Ranging Study*

In seven men, intra-brachial substance P was administered in incremental doubling doses from 0.5 to a maximum of 128 pmol/min for 6 minutes at each dose and was

followed by a 30 minute saline infusion. Venous samples were taken at baseline, following 2 pmol/min, 16 pmol/min and the maximal dose of substance P, and after the final 30 minute saline infusion.

5.3.3.2 *Local Forearm Study*

Twelve men were given intra-arterial doubling doses of substance P at 2, 4 and 8 pmol/min for 10 minutes at each dose, and sodium nitroprusside at 2, 4 and 8 μg/min for 10 minutes at each dose, separated by a 30 minute saline infusion. Substance P and sodium nitroprusside were given single blind, in randomised order. Venous samples were obtained at the end of each period of saline infusion and with each dose of substance P and sodium nitroprusside.

5.3.4 Data Analysis And Statistics

Data were examined, where appropriate, by two way analysis of variance (ANOVA) with repeated measures and two tailed paired Student's *t*-test using Excel *v*4.0 (Microsoft). All results are expressed as means ± standard errors of the mean. Statistical significance was taken at the 5% level.

5.4 RESULTS

All subjects were normotensive (Tables 5.1 and 5.2) and had a normal fasting lipid profile with a mean total cholesterol concentration of 3.90 ± 0.16 mmol/l (150 ± 6 mg/dl), high density lipoprotein cholesterol concentration of 1.02 ± 0.05 mmol/l (39 ± 2 mg/dl) and triglyceride concentration of 0.87 ± 0.08mmol/l (77 ± 7 mg/dl).

5.4.1 Dose ranging study

There were no significant changes in arterial pressure or heart rate throughout the study. Substance P caused an increase in blood flow of the infused forearm ($p<0.001$) from a baseline of 3.7 ± 0.7 mL/100 mL/min to a maximum of 22 ± 2.4 mL/100 mL/min at 64 pmol/min in a dose-dependent manner (Figure 5.1). Five subjects received 64 pmol/min and two received 128 pmol/min as the maximum dose, further infusion of substance P being discontinued because of forearm skin edema and facial flushing. There was a significant increase in the blood flow of the contralateral, non-infused arm ($p=0.001$, ANOVA) which was apparent from 16 pmol/min ($p=0.05$) The relative percentage increase in blood flow of the infused compared with the non-infused arm was dose-dependent, peaking at 32 pmol/min before declining at 64 pmol/min (Figure 5.1).

Substance P caused increases in plasma t-PA antigen and activity concentrations in the infused ($p<0.001$ for both) and non-infused arm ($p=0.003$ for both) which were dose-dependent (Figure 5.2). Plasma from the infused arm demonstrated significantly greater increases in both t-PA activity and antigen concentrations than the non-

Table 5.1
Dose ranging study: systemic haemodynamics, forearm blood flow, estimated net t-PA antigen and activity release, and vWf concentrations and factor VIII:C activity in the infused and non-infused forearms at baseline and during substance P infusion (n=7).

			Substance P Infusion (pmol/min)		
		Baseline	**2**	**16**	**64**
Blood Pressure (mmHg)	**Systolic**	140 ± 6	139 ± 4	139 ± 4	138 ± 7
	Diastolic	70 ± 4	68 ± 5	69 ± 6	65 ± 4
Heart Rate (/min)		57 ± 4	56 ± 4	61 ± 3	63 ± 3
Percentage Change in Forearm Blood Flow		-	233 ± 59 %	466 ± 197 %	221 ± 105 %‡
Absolute Forearm Blood Flow (mL/100 mL/min)	**Non-infused Arm**	3.4 ± 0.4	3.6 ± 0.5	4.6 ± 0.9*	8.8 ± 1.7‡
	Infused Arm	3.7 ± 0.7	11.7 ± 1.5	20.9 ± 2.8	21.5 ± 2.4‡
Estimated Net Release	**t-PA Antigen** (ng/100mL/min)	0.7 ± 0.8	3.8 ± 3.1	18.2 ± 5.3	78.4 ± 25.3‡
	t-PA Activity (IU/100mL/min)	-0.1 ± 0.1	0.9 ± 0.6	20.0 ± 7.8	45.6 ±9.6‡
von Willebrand Factor (IU/mL)	**Non-infused Arm**	0.72 ± 0.06	0.84 ± 0.07	0.99 ± 0.04	0.89 ± 0.04§
	Infused Arm	0.81 ± 0.07	0.93 ± 0.09	1.04 ± 0.15	1.02 ± 0.15
Factor VIII:C (IU/mL)	**Non-infused Arm**	0.49 ± 0.05	0.56 ± 0.05	0.58 ± 0.05	0.53 ± 0.05
	Infused Arm	0.52 ± 0.03	0.57 ± 0.04	0.56 ± 0.05	0.49 ± 0.03

* p=0.05 (*t*-test); ‡ p<0.001 (ANOVA); § p=0.06 (ANOVA)

Table 5.2
Local forearm study: systemic haemodynamics, forearm blood flow, estimated net t-PA antigen and activity release, and vWf concentrations and factor VIII:C activity in the infused and non-infused forearms at baseline and during sodium nitroprusside and substance P infusion (n=12).

		Sodium Nitroprusside (μg/min)					Substance P (pmol/min)				
		0	2	4	8		0	2	4	8	
Blood Pressure (mmHg)	**Systolic**	132 ± 4	133 ± 4	135 ± 4	133 ± 4		131 ± 3	138 ± 4	135 ± 3	136 ± 3	
	Diastolic	70 ± 2	70 ± 2	69 ± 2	68 ± 1		70 ± 2	71 ± 3	70 ± 2	68 ± 3	
Heart Rate (/min)		63 ± 2	61 ± 2	61 ± 2	61 ± 2		64 ± 2	61 ± 2	61 ± 2	60 ± 2	
Percentage Change in Forearm Blood Flow		-	247 ± 53	370 ± 73	541 ± 111	*	-	193 ± 41	286 ± 77	383 ± 79	*
Absolute Forearm Blood Flow (mL/100 mL/min)	**Non-infused Arm**	4.1 ± 0.5	4.1 ± 0.6	3.8 ± 0.6	3.8 ± 0.6		3.8 ± 0.6	4.0 ± 0.6	4.0 ± 0.6	3.9 ± 0.7	
	Infused Arm	4.6 ± 0.7	11.6 ± 0.8	14.8 ± 1.1	18.1 ± 1.4	*	4.4 ± 0.6	11.1 ± 1.2	13.5 ± 1.4	16.5 ± 1.7	*
Estimated Net Release	**t-PA Antigen** (ng/100 mL/min)	-0.5 ± 0.3	-1.6 ± 0.8	-0.5 ± 1.4	0.5 ± 2.7		0.0 ± 0.2	2.9 ± 1.3	7.3 ± 2.1	15.6 ± 3.7	*
	t-PA Activity (IU/100 mL/min)	-0.2 ± 0.1	-0.8 ± 1.0	-0.7 ± 1.7	-1.1 ± 3.5		0.3 ± 0.2	5.0 ± 1.7	8.8 ± 2.5	17.8 ± 3.9	*
von Willebrand Factor (IU/mL)	**Non-infused Arm**	0.66 ± 0.08	0.60 ± 0.07	0.64 ± 0.10	0.68 ± 0.08		0.65 ± 0.10	0.59 ± 0.08	0.61 ± 0.10	0.60 ± 0.13	
	Infused Arm	0.64 ± 0.08	0.62 ± 0.09	0.65 ± 0.09	0.67 ± 0.09		0.62 ± 0.07	0.58 ± 0.09	0.58 ± 0.1	0.63 ± 0.08	
Factor VIII:C (IU/mL)	**Non-infused Arm**	0.61 ± 0.08	0.57 ± 0.08	0.59 ± 0.08	0.62 ± 0.08		0.57 ± 0.09	0.60 ± 0.08	0.59 ± 0.09	0.60 ± 0.09	
	Infused Arm	0.64 ± 0.07	0.64 ± 0.09	0.65 ± 0.09	0.67 ± 0.09		0.53 ± 0.08	0.57 ± 0.10	0.57 ± 0.10	0.63 ± 0.09	

* $p<0.001$ (ANOVA).

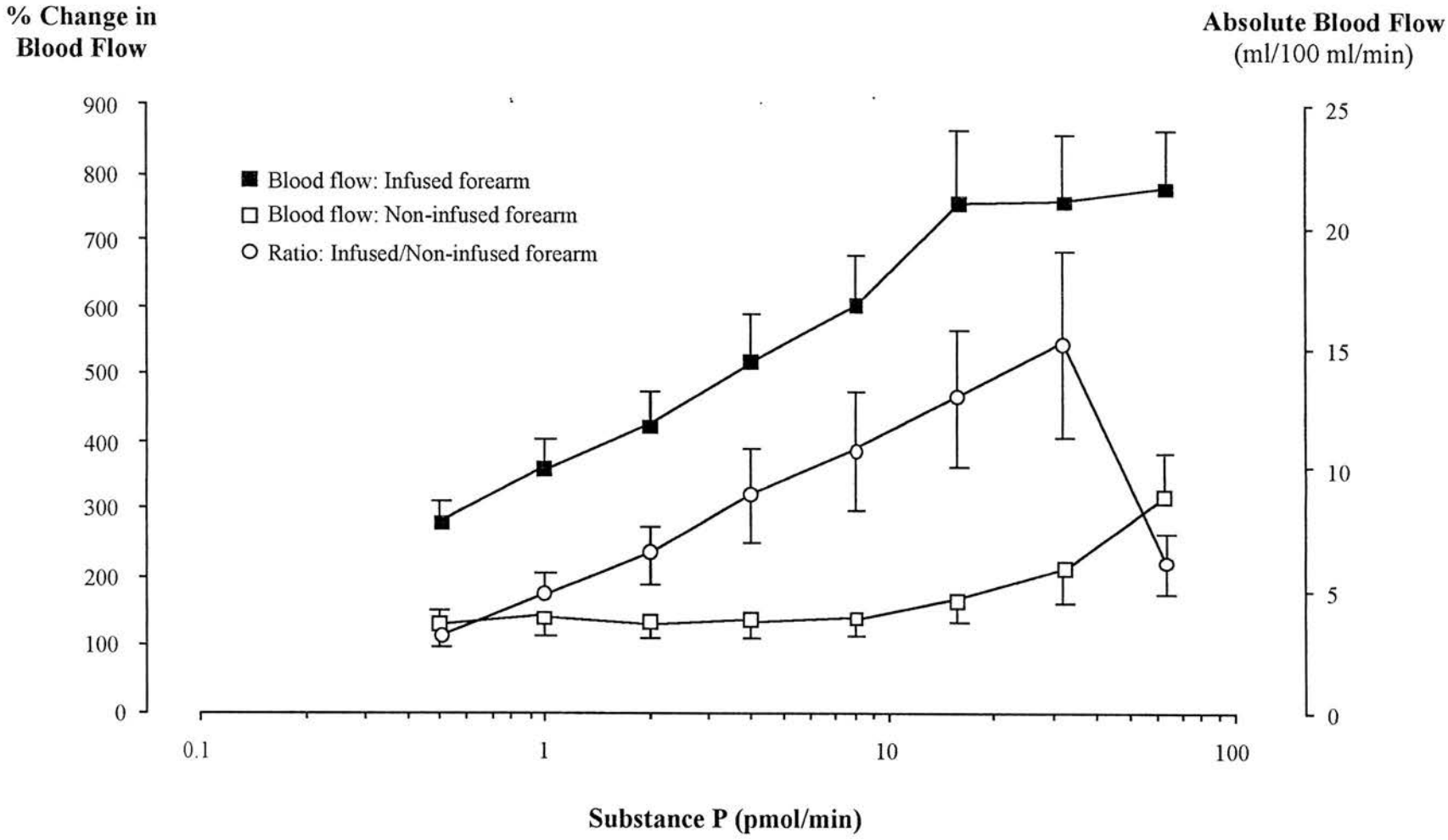


Figure 5.1
Dose ranging study: percentage change and absolute forearm blood flow responses to incremental doses of substance P (n=7).

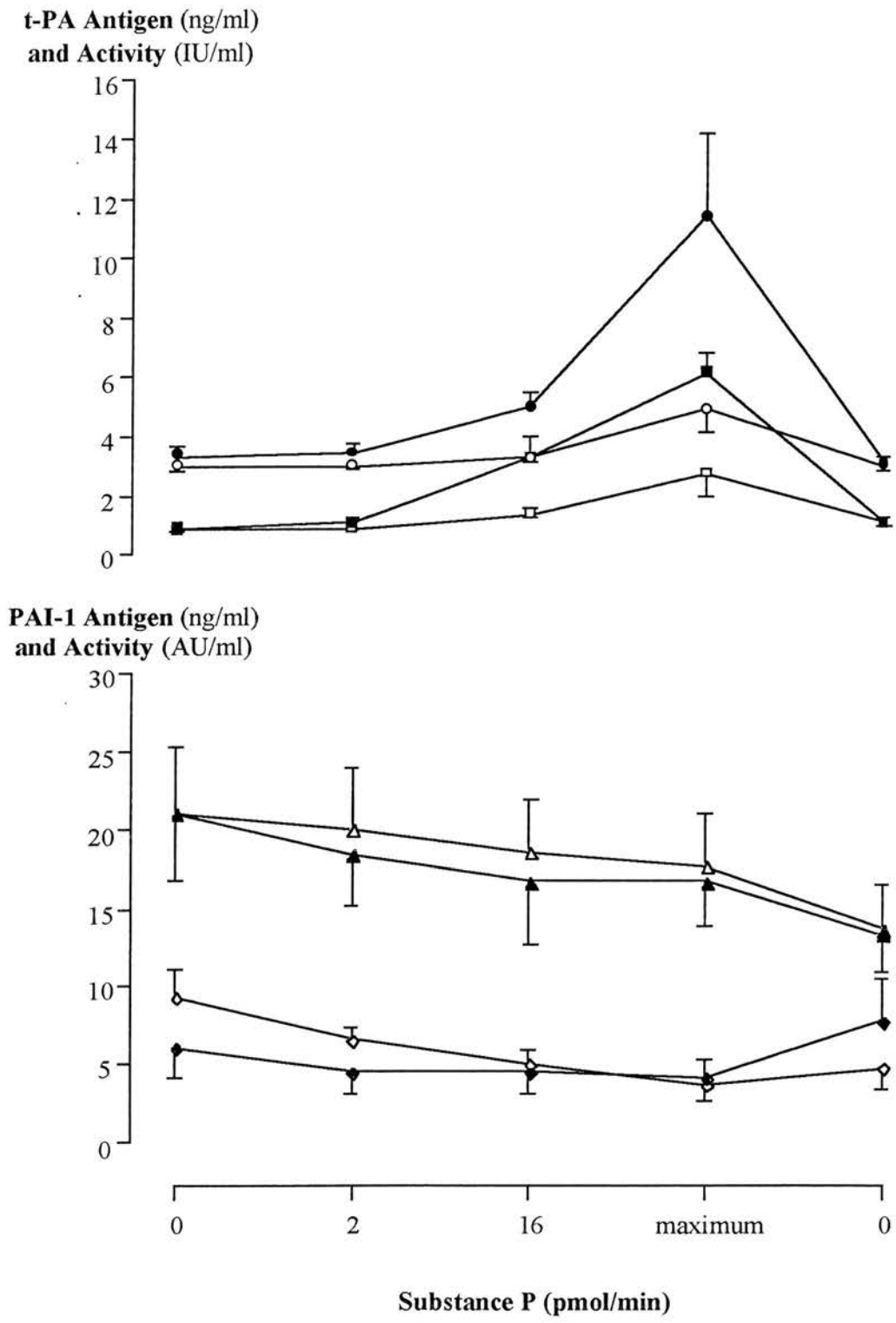


Figure 5.2

Dose ranging study: venous plasma tissue plasminogen activator (t-PA) antigen (circles) and activity (squares), and plasminogen activator inhibitor type 1 (PAI-1) antigen (triangles) and activity (diamonds) concentrations in the infused (closed symbols) and non-infused (open symbols) forearms in response to substance P infusions (n=7). Maximum substance P dose was 64 pmol/min in 5 subjects and 128 pmol/min in 2 subjects. $p<0.001$ for all t-PA concentrations (ANOVA)

infused arm ($p<0.001$). At the maximal dose, mean t-PA activity increased by 630% in the infused arm and 210% in the non-infused arm, whilst mean t-PA antigen increased by 240% and 62% respectively.

There were no significant or consistent changes in plasma PAI-1 antigen or activity concentrations in the infused arm. There was a significant decrease in the plasma PAI-1 activity in the non-infused arm ($p=0.03$) although PAI-1 antigen concentrations did not change significantly ($p=0.64$). There were no significant changes in plasma vWf concentration or factor VIII:C activity in either arm (Table 5.1).

5.4.2 Local Forearm Study

There were no significant changes in blood pressure, heart rate or forearm blood flow in the contralateral arm throughout the study (Table 5.2).

Both substance P and sodium nitroprusside caused selective increases in forearm blood flow in the infused arm ($p<0.001$ for both) in a dose-dependent manner (Table 5.2). Substance P caused a selective and dose-dependent increase in the estimated net release ($p<0.001$ for both) and venous plasma concentrations ($p<0.001$ for both) of both t-PA activity and antigen (Table 5.2; Figure 5.3). In contrast, there were no significant changes in plasma t-PA activity or antigen concentrations in the non-infused arm, or in PAI-1 antigen and activity, vWf or factor VIII:C concentrations in either arm (Table 5.2; Figure 5.3). There were no significant

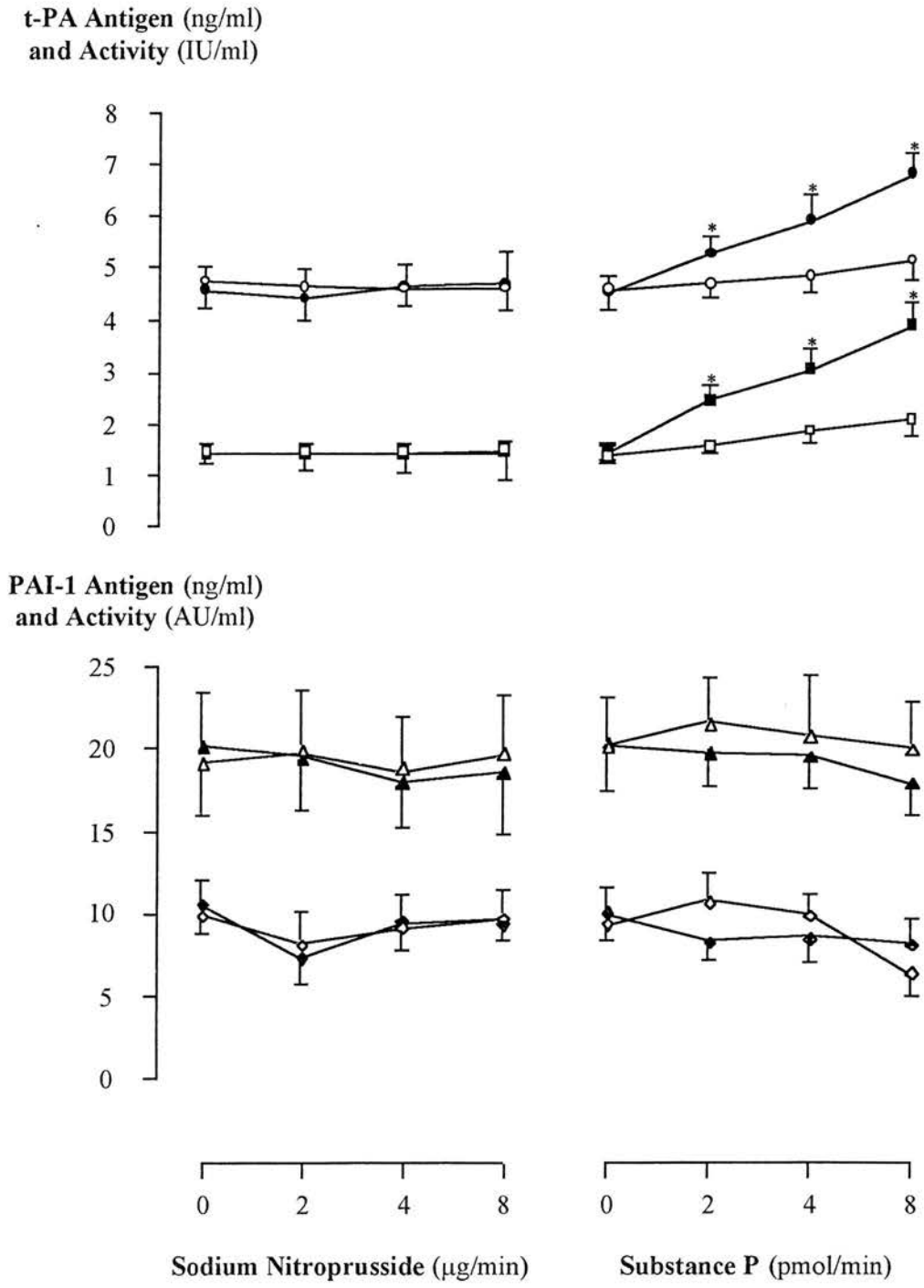


Figure 5.3
Local forearm study: venous plasma tissue plasminogen activator (t-PA) antigen (circles) and activity (squares), and plasminogen activator inhibitor type 1 (PAI-1) antigen (triangles) and activity (diamonds) concentrations in the infused (closed symbols) and non-infused (open symbols) forearms in response to sodium nitroprusside and substance P infusions (n=12). * $p<0.001$ (ANOVA).

changes in t-PA, PAI-1, vWf or factor VIII:C in either arm during sodium nitroprusside infusion (Table 5.2; Figure 5.3).

5.5 DISCUSSION

We have shown, for the first time, that intra-arterial substance P administration causes acute, selective and substantial t-PA release *in vivo* in man. At both systemic and locally active doses, substance P causes t-PA release from the forearm vascular bed without significant effects on the release of PAI-1, vWf and factor VIII:C. This model provides a selective *in vivo* method of assessing acute t-PA release from the endothelium in man.

Intra-brachial substance P has been previously shown to induce local fibrinolysis in the forearm [Fanciullacci *et al* 1993] although the mechanism of this effect had not been determined. However, taken together with our findings, it is apparent that this enhancement of fibrinolytic activity is, at least in part, mediated through t-PA release. Previously, bradykinin was thought to be one of the most potent agents causing t-PA release in animals [Emeis 1992; Tranquille *et al* 1992] and man [Emeis 1992; Brown *et al* 1995]. However, a recent study in man [Brown *et al* 1999] using systemic intravenous bradykinin administration at doses of up to 380 pmol/kg/min, did not show a significant release of t-PA antigen except in the presence of angiotensin converting enzyme inhibition and alterations in systemic haemodynamic parameters. Jern and colleagues [Jern *et al* 1994] have shown a significant net local release of t-PA antigen and activity in response to intra-brachial methacholine. However, a significant increase in venous concentrations of t-PA antigen was not detected and, although measurement of arteriovenous differences should enhance the accuracy of assessing local tissue release, there were no significant increases in the

arteriovenous gradients of t-PA antigen or activity. Indeed, the clearest changes were observed in the arterial t-PA activity which should have remained constant, suggesting that there was systemic stimulation of t-PA release in this study.

Venous plasma t-PA concentrations obtained from a given tissue bed are composed of three components; circulating arterial t-PA, basal or constitutive endothelial cell release of t-PA and facultative or stimulated endothelial cell release of t-PA. The net tissue release of t-PA is equivalent to the product of the plasma flow through the tissue and the arteriovenous difference in plasma t-PA concentrations across it. In the absence of endothelial cell stimulation, but with an increase in blood flow across the tissue bed, venous plasma t-PA concentrations would be expected to fall secondary to a dilutional effect. However, this ignores the potential for clearance of t-PA across the vascular bed [Hajjar 1991], and stimulation of its release by shear stress and flow [Diamond *et al* 1990; Iba *et al* 1991]. Without measuring the arteriovenous concentration gradient across the forearm, net tissue release can only be derived and estimated. However, arterial sampling requires the insertion of large bore cannulae which do not lend themselves to multiple cannulations within the same subject. There is also the potential to introduce artefact from the presence of a larger thrombogenic surface given that activated factor Xa is the most potent stimulant for t-PA release yet known [Emeis 1992]. Rather than assessing arteriovenous differences, we have compared venous plasma t-PA concentrations between infused and non-infused arms and have used very fine gauge arterial cannulae for drug administration only. This method may potentially underestimate the net release of t-PA and fail to detect a modest effect due to the potential flow-dependent, dilutional

changes in venous concentrations. However, typical resting arteriovenous differences are only ~10% of the total venous concentration [Gough *et al* 1992; Jern *et al* 1994] and the basal constitutive release of t-PA antigen is ~0.9 ng/100 mL of tissue/min in the forearm [Jern *et al* 1994]. Thus, in the presence of large increases in t-PA release, the dilutional effect of increased blood flow on constitutive t-PA release will be reduced. Indeed, using this bilateral venous sampling methodology, we have been able to demonstrate a substantial, dose-dependent release of t-PA from the forearm vascular bed in response to substance P infusion. Moreover, despite *in vitro* evidence that t-PA release may be influenced by shear stress [Diamond *et al* 1990; Iba *et al* 1991], we have found that the endothelium-independent nitric oxide donor, sodium nitroprusside, has no significant effect on the venous t-PA concentrations despite comparable increases in blood flow to those with substance P. Sodium nitroprusside is known to have no direct effect on the endothelial cell release of t-PA and PAI-1 *in vitro* [Pannocchia *et al* 1996] and, therefore, it is likely that either shear stress and flow-dependent stimulation of endothelial cell t-PA release is counterbalanced by the potential dilutional effects of increased flow, or that this theoretical flow-dependence of venous concentrations is negligible. This also indicates that increases in nitric oxide and blood flow are not sufficient in themselves to release t-PA from the endothelium. However, it remains a possibility that the L-arginine:nitric oxide pathway plays a role in substance P-induced t-PA release and requires further studies using a combined infusion of substance P and a nitric oxide synthase inhibitor such as L-N^G-monomethyl arginine.

Although we have produced substantial increases in both t-PA activity and antigen, we did not detect release of PAI-1, or the coagulation factors, vWf and factor VIII:C. This would indicate that these agents are not stored in a rapidly translocatable pool within the endothelial cells of the forearm vascular bed or that they are not released in response to substance P over the time course and at the doses used here. However, protracted endothelial cell stimulation may release these factors [Ludlam *et al* 1980; Ridker *et al* 1993b]. In this respect, it is interesting to note the time-dependent reduction in PAI-1 concentrations seen in our studies, although these only achieved statistical significance in the non-infused arm during the dose ranging study. The most likely explanation for a reduction in PAI-1 is that the released and active t-PA is complexed by circulating PAI-1 and subsequently cleared from the circulation by the liver [Otter *et al* 1992]. Thus, substantial local t-PA release will tend to reduce systemic PAI-1 concentrations in the short term, as seen with the administration of pharmacological doses of t-PA [Lucore *et al* 1988].

As anticipated, local substance P infusion did not affect the rate of release of hepatically derived factor VIII:C. In contrast, it is perhaps surprising that we did not observe a rise in plasma vWf concentrations to accompany the release of t-PA. To date, stimulation of t-PA release using a wide range of secretagogues such as thrombin, vasopressin, bradykinin, histamine and desmopressin, has invariably been accompanied by concomitant vWf release [Ludlam *et al* 1980; Levin *et al* 1984; Casonato *et al* 1992]. However, we were unable to detect an acute local release of vWf even in the presence of high local concentrations of substance P in the dose ranging study. This novel selectivity suggests that the endothelium is able to mobilise

different cytoplasmic storage pools in response to specific (NK_1) receptor stimulation. Further studies with more prolonged infusions of substance P would be required to determine whether the endothelial cell vWf or PAI-1 release is delayed or is truly not influenced by substance P.

In summary, brief, locally active and subsystemic infusions of intra-arterial substance P produce a rapid and substantial increase in plasma t-PA activity and antigen concentrations across the forearm bed which appear to act via a flow and nitric oxide independent mechanism. This model provides a powerful method of assessing the *in vivo* capacity of the endothelium to acutely release t-PA within the forearm vascular bed and would be applicable to the assessment of diseases associated with endothelial dysfunction, such as hypercholesterolaemia [Stroes *et al* 1995].

CHAPTER 6

ENDOTHELIN-1 DOES NOT CONTRIBUTE TO THE RELEASE OF TISSUE PLASMINOGEN ACTIVATOR *IN VIVO* IN MAN

Newby DE, Strachan FE, Johnston NR, Webb DJ.
Endothelin-1 does not contribute to the release of tissue plasminogen activator in vivo in man.
Fibrinolysis Proteol 1999;**13**:185-191.

6.1 SUMMARY

Endothelin-1 is a potent endothelium-derived vasoconstrictor peptide with autocrine and paracrine actions. Tissue plasminogen activator (t-PA) and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1), are also released from the vascular endothelium and play a pivotal role in endogenous fibrinolysis. We, therefore, examined the effects of exogenous and endogenous endothelin-1 on t-PA and PAI-1 release *in vivo* in man. Unilateral brachial artery infusions of endothelin-1 at 2.5 and 10 pmol/min, and the selective endothelin type B (ET_B) receptor antagonist, BQ-788, at 1 nmol/min were administered. Blood flow and plasma fibrinolytic factors were measured in both forearms using venous occlusion plethysmography and venous blood samples withdrawn from the antecubital fossae. Endothelin-1 caused a slow onset dose-dependent forearm vasoconstriction ($p<0.001$) with a maximal reduction in blood flow of 40 ± 4% and 63 ± 3% at 2.5 and 10 pmol/min respectively. BQ-788 also caused a slow onset reduction in forearm blood flow ($p<0.001$) reaching a maximum of 21 ± 3%. However, BQ-788 and endothelin-1 did not affect plasma concentrations of t-PA or PAI-1 in the venous effluent of the infused forearm. Despite sustaining significant vasoconstriction, neither endogenous nor exogenous endothelin-1 influences the release of t-PA or PAI-1 in the forearm vascular bed of man. This suggests that endothelin-1 does not provide a major contribution to the regulation of endogenous fibrinolysis in man.

6.2 INTRODUCTION

Endothelin-1 is a potent endothelium-derived vasoconstrictor peptide with autocrine and paracrine actions. It is continuously released by the endothelium and contributes to the maintenance of basal vascular tone [Haynes *et al* 1994] and blood pressure [Haynes *et al* 1996]. There are two main endothelin receptor subtypes, ET_A and ET_B, but only the ET_B receptors are present on the endothelium. Endothelin-1 causes vasoconstriction mainly through stimulation of the smooth muscle cell ET_A receptor, although smooth muscle ET_B receptors may also contribute in some vessel types. This vasoconstrictor response is modulated by autocrine endothelial cell ET_B receptor-mediated generation of the endothelium-derived vasodilators, nitric oxide and prostacyclin.

Following an acute myocardial infarction, plasma endothelin-1 concentrations are elevated and provide an important prognostic marker of survival at one year [Omland *et al* 1994]. Furthermore, on the basis of *in vitro* studies, it has been suggested that endothelin-1 may contribute to the regulation of endogenous fibrinolysis and t-PA release [Lidbury *et al* 1990; Pruis *et al* 1990; Yamamoto *et al* 1992]. However, the evidence is contradictory, with endothelin-1 being found to either inhibit [Yamamoto *et al* 1992] or stimulate [Lidbury *et al* 1990; Pruis *et al* 1990] endothelial cell t-PA release. The role of endothelin-1 in the regulation of endogenous fibrinolysis in man is currently unknown.

We [Newby *et al* 1997b; Newby *et al* 1998] and others [Jern *et al* 1994a+b; Jern *et al* 1997a] have shown, using bilateral forearm venous occlusion plethysmography and unilateral brachial artery infusions, that the forearm release of t-PA and PAI-1 can be determined *in vivo* in man. Therefore, the aim of the current study was, using synthetic endothelin-1 peptide and the selective ET_B receptor antagonist, BQ-788, to determine whether endothelin-1, of exogenous or endogenous origin, acts via the endothelial ET_B receptor to regulate the release of t-PA or PAI-1 *in vivo* in man.

6.3 METHODS

6.3.1 Subjects

Fourteen healthy men aged between 20 and 33 years participated in three studies which were undertaken with the approval of the local Research Ethics Committee and in accordance with the Declaration of Helsinki.

6.3.2 Drugs

Endothelin-1 (Clinalfa AG, Läufelfingen, Switzerland) and BQ-788 (American Peptide Company, Sunnyvale, USA) were administered following dissolution in saline.

6.3.3 Study Design

On each study day, subjects attended fasted and rested recumbent throughout the study. Strain gauges and cuffs were applied and the brachial artery of the non-dominant arm cannulated. Throughout each of the studies, measurements of forearm blood flow were made every 10 minutes. Before drug administration, saline was infused for 30 minutes to allow time for equilibration and the final blood flow measurement during saline infusion was taken as the basal forearm blood flow.

Eight subjects received an intra-brachial infusion of endothelin-1 at 2.5 and 10 pmol/min for 120 minutes given in random order, on two separate occasions, at least one week apart. Eight subjects (two had also attended for endothelin-1 infusions) received an intra-brachial infusion of BQ-788 at 1 nmol/min for

120 minutes. Venous samples were withdrawn from each arm at baseline and at 10, 20, 30, 50, 80 and 120 minutes after the start of endothelin-1 or BQ-788 infusion.

6.3.4 Data Analysis And Statistics

Data were examined by two-way analysis of variance (ANOVA) with repeated measures and two tailed paired Student's *t*-test using Excel *v*5.0 (Microsoft) where appropriate. All results are expressed as mean ± standard error of the mean. Statistical significance was taken at the 5% level. Based on previous data, [Newby *et al* 1997b; Newby *et al* 1998] the study had 90% power to detect a 20% change in plasma t-PA concentrations between treatment periods at the 5% level.

6.4 RESULTS

All subjects were normotensive and there were no significant changes in blood pressure, heart rate or blood flow in the contralateral arm throughout any of the studies (Table 6.1). Haematocrit decreased slightly in each endothelin study (Table 6.1). Between the three protocols there were no significant differences in the baseline values of blood pressure, heart rate, forearm blood flow, haematocrit or plasma concentrations of t-PA and PAI-1.

6.4.1 Endothelin-1 Infusions

Endothelin-1 decreased blood flow in the infused arm ($p<0.001$) in a dose-dependent manner (Figure 6.1) reaching a minimum of 2.5 ± 0.3 mL/100 mL/min at 2.5 pmol/min and 1.6 ± 0.1 mL/100 mL/min at 10 pmol/min, after 120 minutes. This corresponds to a relative reduction in forearm blood flow of 40 ± 4% and 63 ± 3% respectively. The plasma concentrations of t-PA and PAI-1 did not change in the infused arm (Figure 6.2) during endothelin-1 infusion at either concentration (p=NS; one-way ANOVA). In comparison to the non-infused arm, there was a trend ($p=0.06$; two-way ANOVA) for the infused forearm plasma t-PA antigen concentration to be greater with 10 pmol/min of endothelin-1. However, there were no significant differences in plasma concentrations of PAI-1 antigen (Table 6.2) or t-PA activity between the forearms.

Table 6.1.
Systemic haemodynamics, forearm blood flow and haematocrit at baseline and after intra-arterial infusion for 120 min

		BQ-788 1 nmol/min			Endothelin-1 2.5 pmol/min			Endothelin-1 10 pmol/min		
		Basal	Final		Basal	Final		Basal	Final	
Blood Pressure (mmHg)	**Systolic**	130 ± 5	134 ± 6		136 ± 3	140 ± 4		133 ± 4	133 ± 4	
	Diastolic	75 ± 4	77 ± 4		72 ± 3	71 ± 3		70 ± 3	73 ± 4	
Heart Rate (/min)		59 ± 3	60 ± 3		62 ± 4	58 ± 4		61 ± 5	62 ± 4	
Absolute Forearm Blood Flow (mL/100 mL/min)	**Non-infused Arm**	3.1 ± 0.4	3.8 ± 0.2		3.4 ± 0.3	3.7 ± 0.5		3.1 ± 0.3	4.1 ± 0.3	
	Infused Arm	4.5 ± 0.7	4.2 ± 0.4	*	4.0 ± 0.4	2.5 ± 0.3	*	3.6 ± 0.4	1.6 ± 0.1	*
Ratio of Infused/Non-infused		1.35 ± 0.15	1.14 ± 0.06	†	1.13 ± 0.03	0.42 ± 0.03	†	1.05 ± 0.07	0.40 ± 0.03	†
Haematocrit		0.41 ± 0.01	0.41 ± 0.01		0.42 ± 0.02	0.40 ± 0.02	†	0.41 ± 0.01	0.40 ± 0.01	†

*p = 0.001 (two-way ANOVA; infused *vs* non-infused)
†p <0.001 (one-way ANOVA)
‡p <0.05 (paired *t*-test; basal *vs* final)

Table 6.2.
Plasma plasminogen activator inhibitor type 1 (PAI-1) concentrations (ng/mL) during endothelin-1 (ET-1) and BQ-788 infusion

		Time (min)						
		Baseline	**10**	**20**	**30**	**50**	**80**	**120**
BQ-788 (1 nmol/min)	**Infused Arm**	39 ± 15	37 ± 13	43 ± 15	41 ± 13	37 ± 12	37 ± 12	29 ± 9
	Non-Infused Arm	38 ± 13	40 ± 14	43 ± 14	41 ± 13	37 ± 13	33 ± 9	32 ± 9
ET-1 (2.5 pmol/min)	**Infused Arm**	28 ± 7	27 ± 5	26 ± 6	26 ± 5	25 ± 5	21 ± 5	20 ± 5
	Non-Infused Arm	27 ± 5	29 ± 6	29 ± 6	27 ± 5	28 ± 7	25 ± 5	23 ± 4
ET-1 (10 pmol/min)	**Infused Arm**	25 ± 5	26 ± 4	27 ± 5	26 ± 5	23 ± 5	21 ± 4	22 ± 5
	Non-Infused Arm	27 ± 5	27 ± 5	26 ± 5	25 ± 4	24 ± 5	23 ± 5	22 ± 4

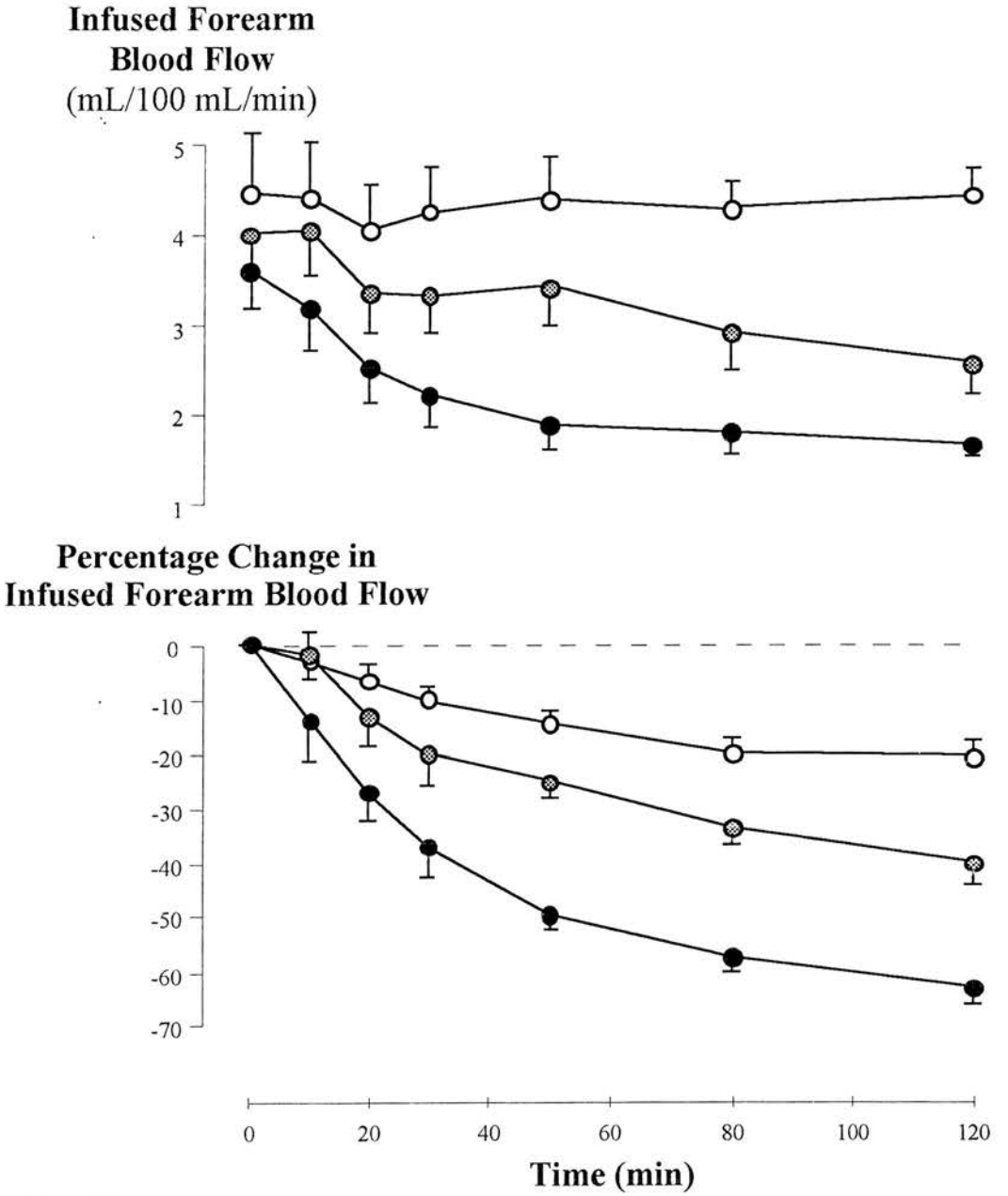


Figure 6.1.
Absolute (mL/100 mL of tissue/min; upper panel) and percentage (% relative to the non-infused forearm, lower panel) change of blood flow in the infused forearm during intra-arterial infusion of BQ-788 (1 nmol/min; ○) and endothelin-1 (2.5 pmol/min; ⊗ and 10 pmol/min; ●).

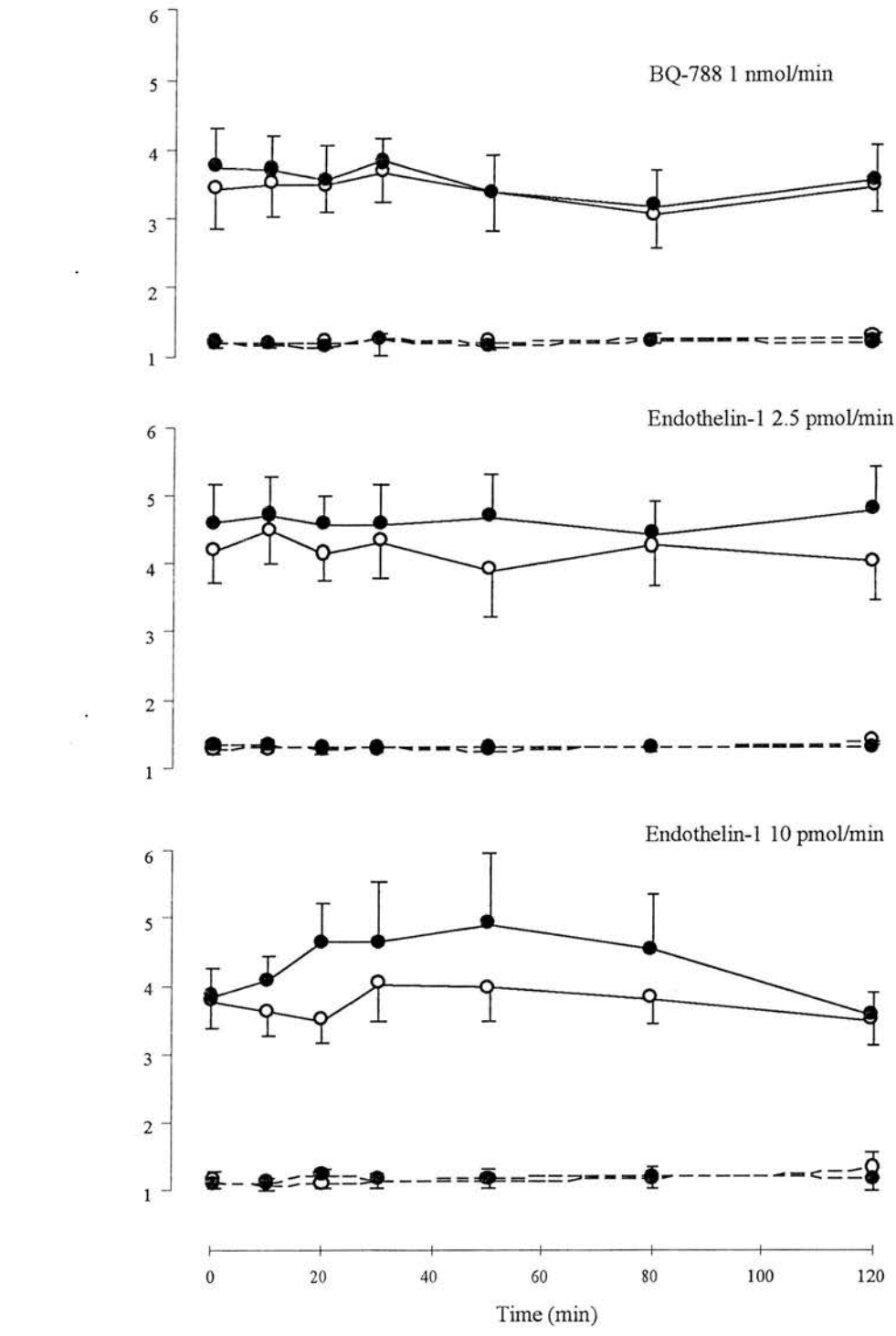


Figure 6.2
Plasma concentrations of tissue plasminogen activator (t-PA) antigen (ng/mL; solid lines) and activity (IU/mL; dashed lines) in the infused (solid circles) and non-infused (open circles) forearm during intra-arterial infusion of BQ-788 (1 nmol/min) and endothelin-1 (2.5 and 10 pmol/min).

6.4.2 BQ-788 Infusion

In comparison to the non-infused arm, BQ-788 decreased blood flow in the infused forearm after 120 minutes (relative reduction of 21 ± 3%) although the absolute blood flow was unchanged (Table 6.1 and Figure 6.1). The plasma concentrations of t-PA and PAI-1 (Table 6.2 and Figure 6.2) did not change in the infused forearm (p=NS; one-way ANOVA) or in comparison to the non-infused forearm (p=NS; two-way ANOVA).

There was no significant net release of t-PA with infusions of either endothelin-1 or BQ-788 (Table 6.3).

Table 6.3.
Estimated net release of tissue plasminogen activator (t-PA) antigen across the forearm during endothelin-1 (ET-1) and BQ-788 infusion
Mean (95% confidence intervals)

		Time (min)						
		Baseline	**10**	**20**	**30**	**50**	**80**	**120**
t-PA Release (ng/100 mL/min)	**BQ-788** (1 nmol/min)	1.1 (-0.1 to 2.3)	0.0 (-1.6 to 1.6)	0.4 (-1.0 to 1.8)	0.3 (-1.5 to 2.1)	-0.2 (-1.8 to 1.4)	0.1 (-1.3 to 1.5)	0.1 (-1.7 to 1.9)
	ET-1 (2.5 pmol/min)	0.1 (-0.7 to 0.9)	1.4 (-0.6 to 3.4)	2.5 (0.5 to 4.5)	1.0 (-0.8 to 2.6)	2.0 (-1.0 to 5.0)	1.7 (-1.1 to 4.5)	0.0 (-0.2 to 0.2)
	ET-1 (10 pmol/min)	1.3 (-0.3 to 2.9)	0.5 (-0.4 to 1.9)	0.9 (0.1 to 1.7)	0.3 (-0.4 to 1.7)	0.9 (-0.1 to 1.9)	0.1 (-0.7 to 0.9)	0.8 (0.0 to 1.6)

6.5 DISCUSSION

We have demonstrated that, despite causing significant reductions in blood flow, neither endogenous nor exogenous endothelin-1 influences the release of t-PA or PAI-1 in the forearm vascular bed of man. This suggests that endothelin-1 does not contribute to the regulation of endogenous fibrinolysis in man.

Endothelial cell culture techniques have limitations in the investigation of t-PA release and may not be truly representative of the *in vivo* function of these cells. The amount of t-PA released in culture is small and necessitates prolonged incubation periods and sensitive assays. Moreover, the phenotype of endothelial cells in culture, and the ability to release t-PA, changes with increasing passages. This may account for the disparity of our findings with previous endothelial cell culture studies [Yamamoto *et al* 1992].

Studies in intact whole animals have suggested that systemic endothelin-1 infusion is associated with stimulation of t-PA release [Lidbury *et al* 1990], although plasma t-PA concentrations are not increased by low sub-pressor doses of endothelin-1 in man [Kapiotis *et al* 1997]. Systemic endothelin-1 administration, particularly at pressor doses, will induce changes in cardiac function and regional blood flow as well as having widespread effects on disparate tissues. Thus, the consequent changes in systemic fibrinolytic parameters will be a combination of many factors, potentially including hepatic production and clearance of t-PA and PAI-1. One approach, to avoid these confounding systemic effects, has been to use the isolated perfused rat

hindlimb model. This *ex vivo* model has been reported to demonstrate that endothelin-1 infusion stimulates modest amounts of t-PA release [Pruis *et al* 1990]. However, this increased 'release' may, in part, reflect the concentrating effects of a reduction in blood flow associated with endothelin-1 infusion and the concentrations of endothelin-1 administered. In studies conducted to date [Lidbury *et al* 1990; Pruis *et al* 1990; Yamamoto *et al* 1992], endothelin-1 has been administered in nanomolar concentrations. Although local abluminal concentrations may be high, normal human plasma endothelin-1 concentrations are in the femtomolar range. Indeed, in the present study, assuming a total forearm blood flow of 30 to 50 mL/min, the forearm tissue concentration of endothelin-1 during the 10 pmol/min infusion will be 200 to 300 fmol/mL. The previous *ex vivo* animal studies [Pruis *et al* 1990], therefore, represent some 4 to 5 orders of magnitude higher concentrations and the release of t-PA is likely to represent a pharmacological rather than physiological effect.

We have not detected a significant release of t-PA from the forearm with endothelin-1 infusion despite a 63% reduction in blood flow at the higher dose. Basal t-PA release is of the order of ~0.9 ng/100 mL of tissue/min in the forearm [Jern *et al* 1994] and the apparent trend for an increase in t-PA antigen concentrations may, in part, reflect the reduction in blood flow associated with the marked forearm vasoconstriction (see Figure 6.3). This is borne out by the unchanged t-PA activity, because it would be anticipated that plasma PAI-1 and t-PA antigen concentrations would increase proportionately with reductions in blood flow. ET_B receptor antagonism causes both inhibition of endothelium-derived vasodilators such as nitric oxide, and potential hyperstimulation of the unopposed ET_A receptor. However, as

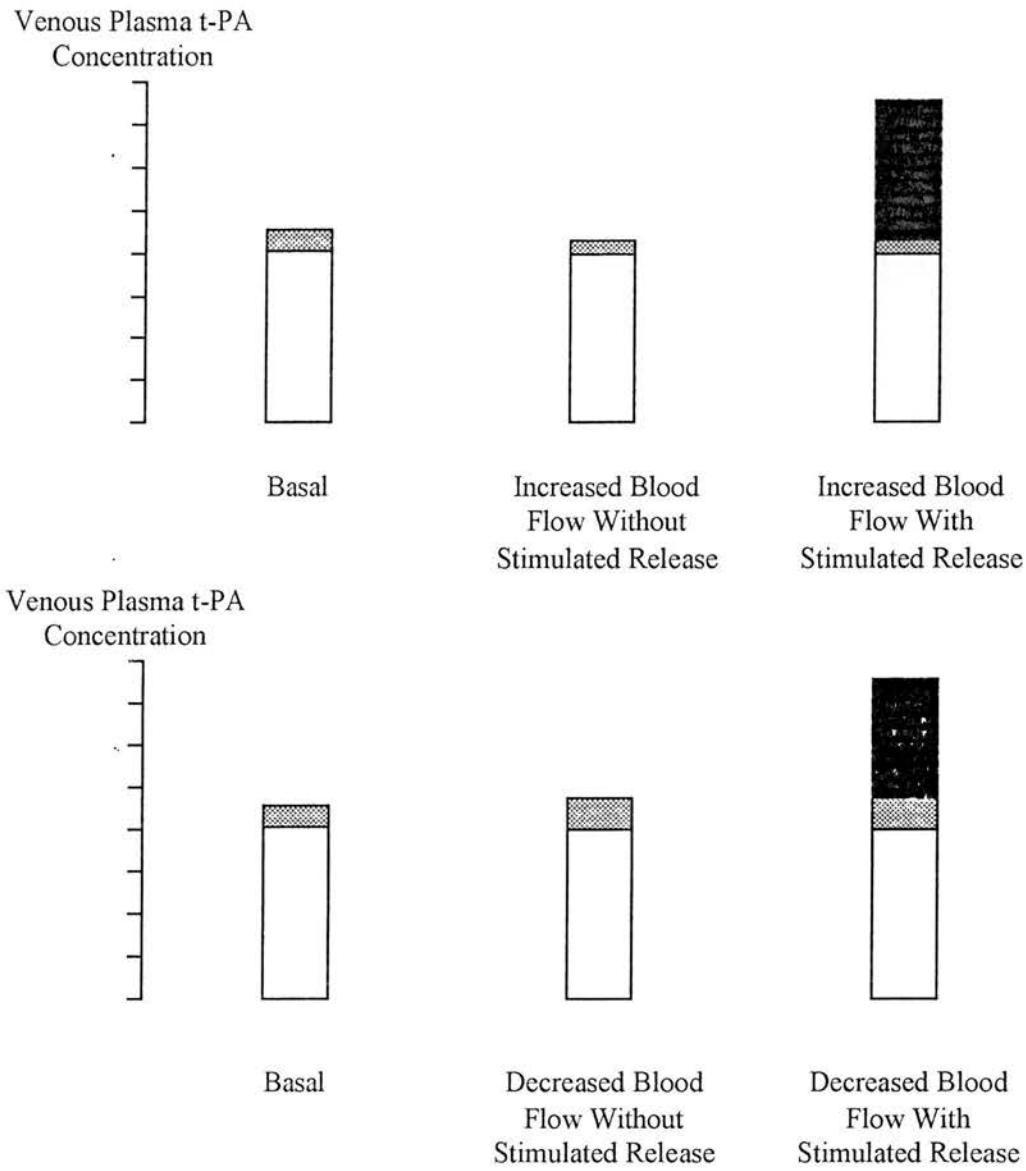


Figure 6.3
Theoretical components of venous plasma t-PA concentration under basal conditions and during increases (upper panel) and decreases (lower panel) in blood flow with and without direct stimulation of t-PA release.
Open bars - circulating or arterial t-PA
Grey bars - basal "constitutive" t-PA released from the tissue bed
Black bars - stimulated "facultative" t-PA released from the tissue bed

with endothelin-1, BQ-788 did not affect plasma concentrations of t-PA or PAI-1 in the infused forearm.

Forearm release of t-PA has been demonstrated using various endothelial cell stimulants including methacholine [Jern *et al* 1994a+b; Jern *et al* 1997], noradrenaline [Jern *et al* 1997] and desmopressin [Wall *et al* 1998]. Using the same technique as in the present study, we have previously demonstrated *in vivo* t-PA release of up to 80 ng/100 mL of tissue/min across the human forearm using intra-brachial substance P infusion [Newby *et al* 1997b] and this release is sustained for at least 2 hours [Newby *et al* 1998]. In contrast, stimulation or antagonism of the endothelial ET_B receptor, with endothelin-1 and BQ-788 respectively, does not appear to influence forearm t-PA release. It is, therefore, unlikely that endothelin-1 provides a major contribution to the regulation of t-PA release in man, although we cannot exclude a small stimulatory effect.

6.5.1 Study Limitations

In the forearm, typical resting arteriovenous differences are only ~10% of the total venous t-PA concentration and the basal constitutive release of t-PA antigen is ~0.9 ng/100 mL of tissue/min [Jern *et al* 1994a]. We have measured venous-venous differences between the infused and non-infused arms which, unlike the measurement of arteriovenous differences of the infused arm, has the disadvantage of not being able to correct for blood flow-dependent changes in venous plasma t-PA concentrations. Theoretically (see Figure 6.3), in the absence of an alteration in t-PA release, a 60% reduction in blood flow would be anticipated to increase total venous

plasma t-PA concentrations by only ~7%, whereas a 200% increase in flow would reduce t-PA concentrations to the same degree (~7%). In the presence of stimulated t-PA release, these small flow-dependent changes are proportionately reduced even further.

The measurement of arteriovenous differences necessitates arterial sampling and the insertion of large bore cannulae (19-G to 20-G) which do not lend themselves to multiple cannulations within the same subject. Moreover, there is also the potential to introduce artefact from the presence of a larger thrombogenic surface, given that activated factor Xa is the most potent stimulant for t-PA release yet known [Emeis 1992]. To minimise arterial trauma and facilitate repeated studies in the same subjects, we have used 27-G arterial cannulae which permit drug infusion but not arterial blood sampling. However, we would suggest that flow-dependent changes in venous t-PA concentrations are small, within the variability of the t-PA assays (~5 to 7%) and are not of practical importance. Interestingly, a significant fall in the arteriovenous difference, or venous plasma concentration, of t-PA has not been detected during blood flow increases of up to 600% with sodium nitroprusside infusion [Jern *et al* 1994a; Newby *et al* 1997b; Stein *et al* 1998].

Measurement of venous-venous and arteriovenous differences both have the potential limitation that they can only estimate the net release of t-PA from the forearm and are unable to take account of clearance of t-PA within the forearm. However, the majority of t-PA is removed from the circulation by the liver [Chandler *et al* 1997]

and the contribution of forearm clearance of t-PA is, therefore, likely to be very small.

During the present study, we did not see changes in heart rate or blood pressure to suggest systemic effects of endothelin-1 [Pernow *et al* 1996] or BQ-788 infusion [Strachan *et al* 1999]. However, measuring venous concentrations bilaterally will control for any potential systemic effects which may go unrecognised if arteriovenous differences are measured in isolation. Once a drug has a systemic rather than a local effect, there is always the concern that subsequent t-PA release may be influenced or mediated by the release of other humoral factors, such as catecholamines. Moreover, if the main mechanism of t-PA release is mediated by a systemically released intermediate factor, then measuring arteriovenous differences could fail to detect this since arterial concentrations may remain unchanged and venous concentrations will rise in both forearms.

CHAPTER 7

THE L-ARGININE:NITRIC OXIDE PATHWAY CONTRIBUTES TO THE ACUTE RELEASE OF TISSUE PLASMINOGEN ACTIVATOR *IN VIVO* IN MAN

Newby DE, Wright RA, Dawson P, Ludlam CA, Fox KAA, Boon NA, Webb DJ.
The L-arginine:nitric oxide pathway contributes to the acute release of tissue plasminogen activator *in vivo* in man.
Cardiovasc Res 1998;**38**:485-492.

7.1 SUMMARY

Effective endogenous fibrinolysis requires rapid release of endothelial tissue plasminogen activator (t-PA). Using the nitric oxide synthase inhibitor, L-N^G-monomethylarginine (L-NMMA), we examined the contribution of endogenous nitric oxide to substance P induced t-PA release *in vivo* in man. Blood flow and plasma fibrinolytic and haemostatic factors were measured in both forearms of eight healthy male volunteers who received unilateral brachial artery infusions of substance P (2 to 8 pmol/min) and L-NMMA (1 to 4 μg/min). Substance P caused dose-dependent increases in blood flow ($p<0.001$) and plasma t-PA antigen ($p=0.04$) and activity ($p<0.001$) concentrations confined to the infused forearm but had no effect on plasminogen activator inhibitor type 1 (PAI-1) or von Willebrand factor concentrations. In the presence of L-NMMA, substance P again caused significant increases in blood flow ($p<0.001$) and t-PA antigen ($p=0.003$) and activity ($p<0.001$) concentrations but these increases were significantly less than with substance P alone ($p<0.001$, $p=0.05$ and $p<0.01$ respectively). L-NMMA alone significantly reduced blood flow in the infused arm, but had no measurable effect on t-PA or PAI-1 concentrations. The L-arginine:nitric oxide pathway contributes to substance P induced t-PA release *in vivo* in man. This provides an important potential mechanism whereby endothelial dysfunction increases the risk of atherothrombosis through a reduction in the acute fibrinolytic capacity.

7.2 INTRODUCTION

Endothelial cell culture techniques have limitations in the investigation of t-PA release and may not be truly representative of the *in vivo* function of these cells. The amount of t-PA released in culture is small and necessitates prolonged incubation periods and sensitive assays. Moreover, the phenotype of endothelial cells in culture, and the ability to release t-PA, changes with increasing passages. In contrast, under *in vivo* physiological conditions, the endothelium is arranged within a non-planar three dimensional vascular bed, has a more favourable volume to surface area ratio, and is exposed to pulsatile blood flow and pressure changes.

We have recently described an *in vivo* model to assess acute t-PA release in man [Newby *et al* 1997b]. Using intra-brachial infusions of substance P, we have shown a dose-dependent release of t-PA from the human forearm without causing significant release of vWf or plasminogen activator inhibitor type 1 (PAI-1). This suggests either a selective action of substance P or the lack of a rapidly translocatable pool of PAI-1 and vWf. However, we have previously used only brief (~10 min) substance P infusions [Newby *et al* 1997b] and protracted stimulation may release these factors [Ludlam *et al* 1980; Tranquille *et al* 1992; Ridker *et al* 1993b].

Substance P causes endothelium-dependent vasodilatation [Gross *et al* 1994] which is mediated by the endothelial cell neurokinin type 1 receptor [Stjärne *et al* 1994] and is, in part, related to the release of nitric oxide [Cockcroft et l 1994; Newby *et al* 1997c; Quyyumi *et al* 1997]. However, because t-PA release is not seen with

infusions of the nitric oxide donor and vasodilator, sodium nitroprusside [Jern *et al* 1994a; Newby *et al* 1997b], an increase in nitric oxide and blood flow together do not release t-PA from the endothelium. Nevertheless, it remains a possibility that the L-arginine:nitric oxide pathway contributes to substance P induced t-PA release.

Therefore, the aims of the current study were 2-fold: first, to ascertain whether prolonged substance P infusion can cause vWf or PAI-1 release; and second, to determine whether nitric oxide synthase inhibition using L-N^G-monomethylarginine (L-NMMA) affects basal or substance P induced t-PA release.

7.3 METHODS

7.3.1 Subjects

Eight healthy men aged between 20 and 33 years participated in three studies which were undertaken with the approval of the local Research Ethics Committee and in accordance with the Declaration of Helsinki.

7.3.2 Drugs

Pharmaceutical-grade substance P (Clinalfa AG, Läufelfingen, Switzerland) and L-N^G-monomethylarginine (L-NMMA; Clinalfa AG) were administered following dissolution in saline.

7.3.3 Study Design

On three separate occasions, at 9.00 am, subjects attended fasted and rested recumbent throughout each study. Strain gauges and cuffs were applied and the brachial artery of the non-dominant arm cannulated. Throughout all protocols, measurements of forearm blood flow were made every 10 minutes. Saline was infused for the first 30 minutes to allow time for equilibration and the final blood flow measurement during saline infusion was taken as the basal forearm blood flow. Thereafter, subjects underwent the following protocols, in random order, each separated by at least one week: *protocol 1*, each subject received intra-arterial substance P at 2, 4 and 8 pmol/min, for 10 minutes at each dose, followed by a continuous infusion of 8 pmol/min for a further 90 minutes; *protocol 2*, L-NMMA

was co-infused at 4 μmol/min for 10 minutes before and throughout the same substance P infusion as protocol 1; and *protocol 3*, subjects received intra-arterial L-NMMA at 1, 2 and 4 μmol/min for 10 minutes at each dose followed by a continuous infusion of 4 μmol/min for a further 90 minutes. Venous samples were withdrawn from each arm at baseline and at 10, 20, 30, 50, 80 and 120 minutes after the start of substance P (for protocols 1 and 2) or L-NMMA infusion (protocol 3).

7.3.4 Data Analysis And Statistics

Data were examined, where appropriate, by two-way analysis of variance (ANOVA) with repeated measures and two tailed paired Student's *t*-test using Excel *v*5.0 (Microsoft). Tachyphylaxis was assessed by comparing the 30 minute (peak) and 120 minute (final) values with a two tailed paired Student's *t*-test. Area under the curve (AUC) was calculated for the estimated net release of t-PA across the study period. All results are expressed as mean ± standard error of the mean. Statistical significance was taken at the 5% level.

7.4 RESULTS

All subjects were normotensive and there were no significant changes in blood pressure, heart rate or blood flow in the contralateral arm throughout any of the studies (Table 7.1). Haematocrit decreased slightly in each study (Table 7.1). Between the three protocols there were no significant differences in the baseline values of blood pressure, heart rate, forearm blood flow, haematocrit or plasma concentrations of t-PA and PAI-1 antigen and activity.

7.4.1 Isolated Infusions Of Substance P And L-NMMA

Substance P increased blood flow in the infused arm ($p < 0.001$) in a dose-dependent manner (Table 7.2; Figure 7.1) reaching a maximum increase of 15.9 ± 1.9 mL/100 mL/min after 10 minutes at 8 pmol/min. Following prolonged infusion, substance P induced vasodilatation demonstrated tachyphylaxis and decreased to 12.1 ± 1.3 mL/100 mL/min after 100 minutes of substance P at 8 pmol/min ($p<0.003$ *vs* 10 minutes). In comparison to the non-infused arm, substance P caused a dose-dependent increase in venous plasma t-PA activity ($p<0.001$) and antigen ($p<0.04$) concentrations of the infused arm which did not undergo significant tachyphylaxis (Figure 7.2). Concentrations of plasma PAI-1 activity were also reduced in the infused arm ($p=0.04$; Figure 7.2). In contrast, there were no significant changes in plasma PAI-1 antigen, vWf or factor VIII:C concentrations in either arm (Table 7.2 and Figure 7.2).

Table 7.1.
Haemodynamics, forearm blood flow and haematocrit at baseline and completion of the 3 study protocols

		Substance P Alone		L-NMMA Alone		Substance P + L-NMMA	
		Basal	**Final**	**Basal**	**Final**	**Basal**	**Final**
Blood Pressure (mmHg)	**Systolic**	137 ± 3	136 ± 3	135 ± 5	136 ± 5	133 ± 6	132 ± 6
	Diastolic	71 ± 2	71 ± 3	68 ± 3	71 ± 3	69 ± 4	70 ± 5
Heart Rate (/min)		65 ± 4	61 ± 3	59 ± 2	58 ± 3	60 ± 3	61 ± 3
Absolute Forearm Blood Flow (mL/100 mL/min)	**Non-infused Arm**	3.1 ± 0.3	3.7 ± 0.5	3.4 ± 0.7	3.6 ± 0.6	3.6 ± 0.5	4.9 ± 0.7
	Infused Arm	3.6 ± 0.4	12.1 ± 1.3 *	3.9 ± 0.7	2.1 ± 0.2 *	4.2 ± 0.7	9.3 ± 1.4 *
Haematocrit		0.439 ± 0.006	0.430 ± 0.007 †	0.430 ± 0.010	0.421 ± 0.013	‡ 0.426 ± 0.008	0.414 ± 0.010 †

*p<0.001; †p<0.005; ‡p=0.02

Table 7.2.
Blood flow and plasma von Willebrand factor and factor VIII:C activity concentrations in both arms during isolated substance infusion: protocol 1

		Time (min)						
		Baseline	**10**	**20**	**30**	**50**	**80**	**120**
Substance P Dose (pmol/min)		0	2	4	8	8	8	8
Absolute Forearm Blood Flow (mL/100 mL/min)	**Non-infused Arm**	3.1 ± 0.3	3.3 ± 0.3	3.4 ± 0.4	3.3 ± 0.3	3.5 ± 0.3	3.8 ± 0.4	3.7 ± 0.5
	Infused Arm	3.6 ± 0.4	11.8 ± 1.8	13.8 ± 1.9	15.9 ± 1.9	14.5 ± 1.7	12.6 ± 1.2	12.1 ± 1.3 *
von Willebrand Factor (IU/mL)	**Non-infused Arm**	0.84 ± 0.13	0.64 ± 0.10	0.85 ± 0.13	0.60 ± 0.09	0.95 ± 0.18	0.80 ± 0.10	1.20 ± 0.16
	Infused Arm	0.73 ± 0.09	0.72 ± 0.15	0.73 ± 0.11	0.86 ± 0.20	0.98 ± 0.16	0.99 ± 0.17	1.03 ± 0.17
Factor VIII:C (IU/mL)	**Non-infused Arm**	0.50 ± 0.06	0.48 ± 0.04	0.51 ± 0.05	0.51 ± 0.05	0.58 ± 0.09	0.65 ± 0.10	0.72 ± 0.09
	Infused Arm	0.50 ± 0.05	0.50 ± 0.05	0.51 ± 0.07	0.52 ± 0.07	0.64 ± 0.07	0.64 ± 0.08	0.69 ± 0.10

*$p<0.001$

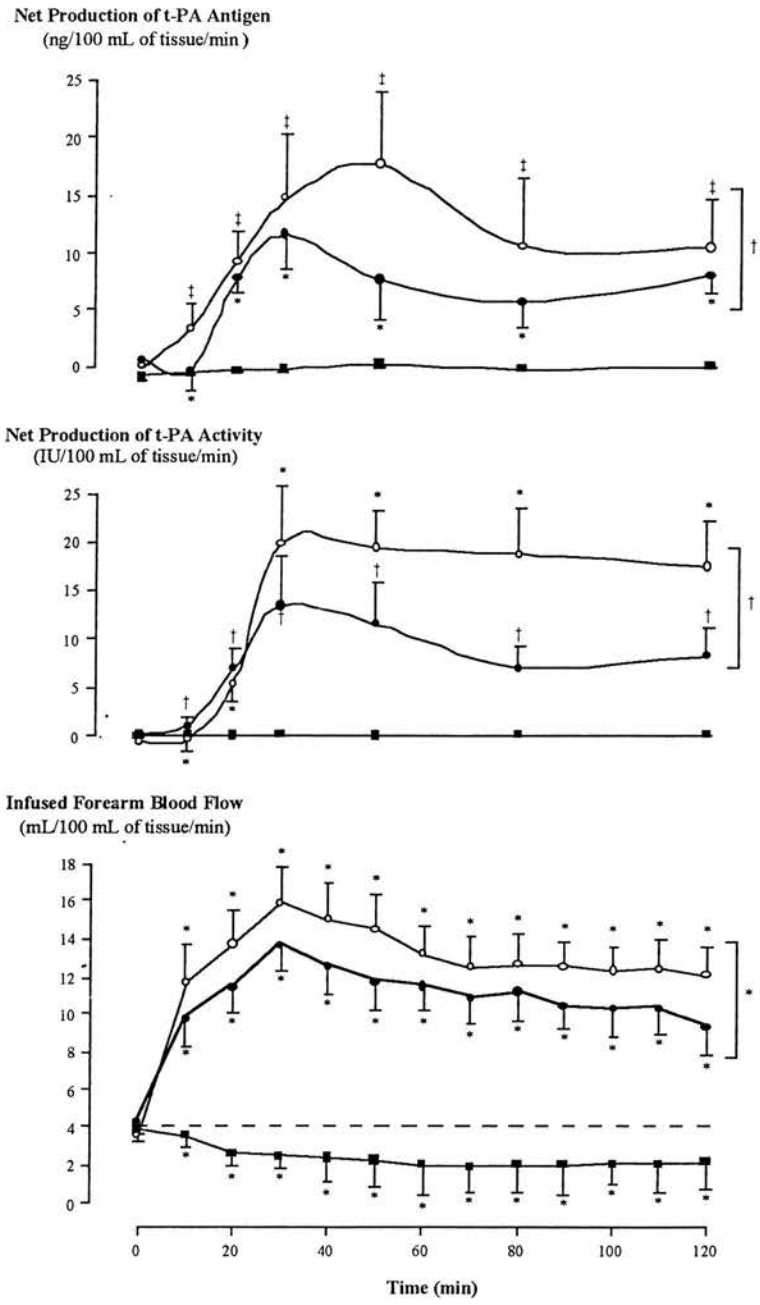


Figure 7.1.
Infused forearm blood flow and estimated net release of t-PA antigen and activity during protocol 1 (substance P alone; o), protocol 2 (substance P and L-NMMA; •) and protocol 3 (L-NMMA alone; ■). *p<0.001; †p=0.05; ‡p=0.09 (ANOVA).

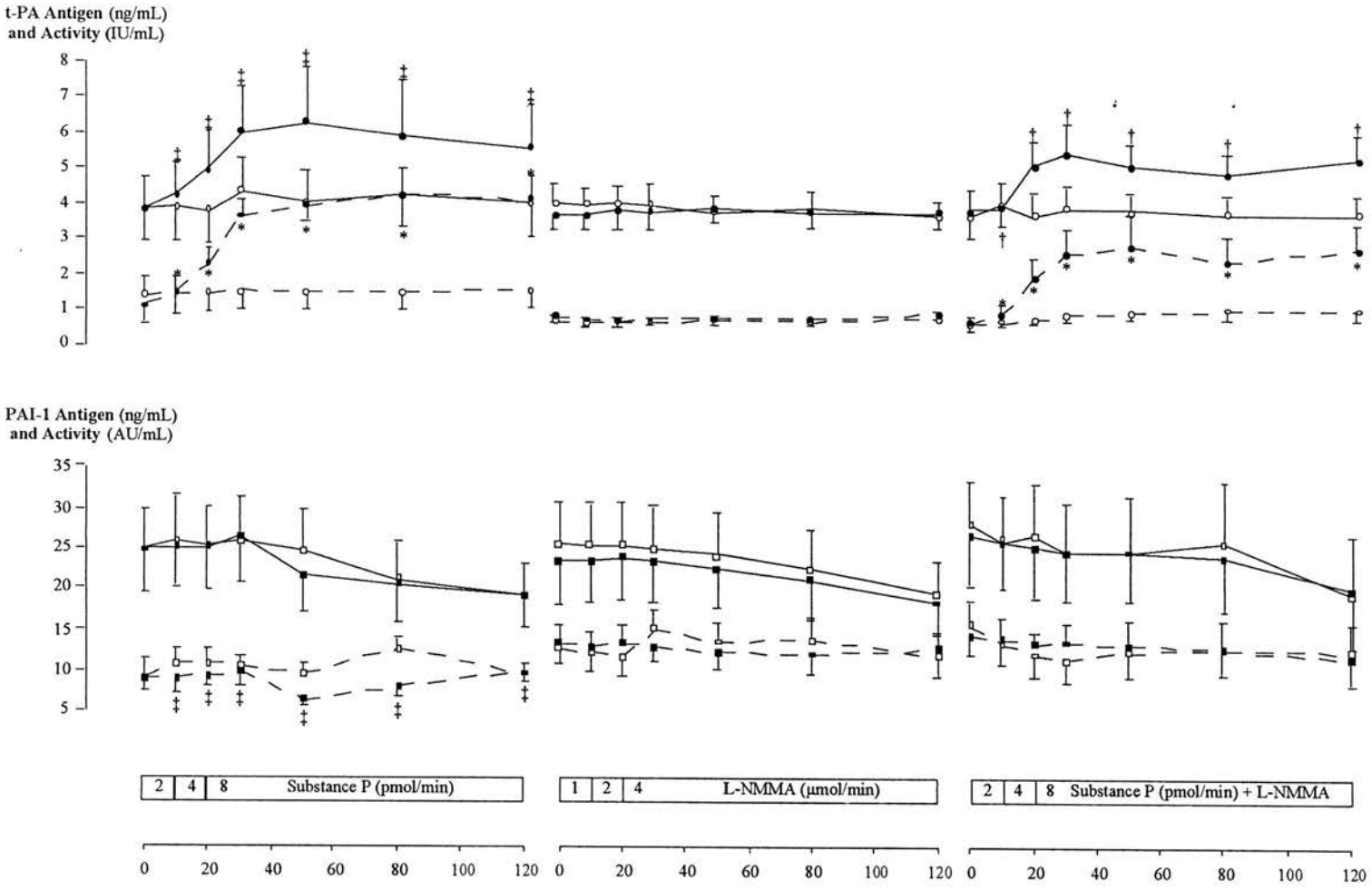


Figure 7.2.

Plasma concentrations of t-PA (circles) and PAI-1 (squares) antigen (solid lines) and activity (dashed lines) in the infused (closed symbols) and non-infused (open symbols) arms in the 3 protocols. *p<0.001; †p<0.003; ‡p=0.04 (ANOVA).

L-NMMA decreased blood flow in the infused arm ($p<0.001$) in a dose-dependent manner (Table 7.2 and Figure 7.1) reaching 2.1 ± 0.2 mL/100 mL/min after 100 minutes at 4 μmol/min. There were no significant changes in the concentrations of plasma t-PA and PAI-1 antigen or activity in either arm during infusion of L-NMMA (Figure 7.2).

7.4.2 Co-infusion Of L-NMMA And Substance P

In the presence of L-NMMA, substance P increased blood flow in the infused arm ($p<0.001$) in a dose-dependent manner (Figure 7.1) reaching a maximum increase of 13.7 ± 1.7 mL/100 mL/min after 10 min at 8 pmol/min. This response underwent tachyphylaxis and decreased to 9.3 ± 1.4 mL/100 mL/min after 100 min of substance P at 8 pmol/min ($p<0.002$ *vs* 10 minutes). In comparison with the non-infused arm, substance P co-infused with L-NMMA caused a dose-dependent increase in plasma t-PA activity ($p<0.001$) and antigen ($p<0.003$) concentrations of the infused arm which did not undergo significant tachyphylaxis (Figure 7.2). L-NMMA caused a significant attenuation of substance P induced increases in blood flow ($p<0.001$) and plasma t-PA activity concentrations ($p<0.003$) in the infused forearm, but not plasma t-PA antigen.

7.4.3 Estimated Net t-PA Production

L-NMMA infused alone had no significant effects on t-PA release: 95% confidence intervals for t-PA antigen and activity release are 0.31 to -0.68 ng/100 mL/min and 0.27 to -0.06 IU/100 mL/min respectively. Substance P caused dose-dependent increases in the estimated net release of t-PA antigen and activity in the presence or

absence of L-NMMA ($p<0.001$) which did not undergo significant tachyphylaxis. However, the magnitude of the increase in release of both t-PA antigen ($p=0.05$) and activity ($p<0.01$) was significantly reduced in the presence of L-NMMA (Figure 7.1). L-NMMA reduced the AUC for the substance P induced release of t-PA antigen and activity by 40% and 46% respectively.

7.5 DISCUSSION

We have shown that intra-brachial substance P infusion increases forearm blood flow and plasma t-PA concentrations for up to 2 hours without a demonstrable effect on plasma PAI-1 or vWf concentrations. Although the nitric oxide synthase inhibitor, L-NMMA, significantly reduced forearm blood flow without affecting basal t-PA release, it inhibited the increases in blood flow, plasma t-PA concentrations and t-PA release produced by substance P administration in the forearm. These data suggest that the L-arginine:nitric oxide pathway contributes to substance P induced t-PA release *in vivo* in man. In contrast, we [Newby *et al* 1997b] and others [Jern *et al* 1994a+b] have shown previously that t-PA release is not seen with the large local increases in nitric oxide delivery and blood flow associated with infusions of the nitric oxide donor, sodium nitroprusside. Taken together, these findings indicate that increases in nitric oxide and blood flow are not sufficient *per se* to release t-PA but, through the L-arginine:nitric oxide pathway, are able to enhance substance P induced t-PA release.

The permissive role of intracellular mediators in the mechanism of t-PA release has been described previously. In the rat perfused hindlimb model, increasing intracellular calcium alone is insufficient to cause t-PA release whilst it is essential for bradykinin induced t-PA release [Tranquille *et al* 1991]. However, the regulation of t-PA release is complex and may involve several signal transduction pathways [Rydholm *et al* 1995]. This is reflected by the diversity of mediators – such as thrombin, bradykinin and desmopressin – which can release t-PA and increase t-PA

activity [Ludlam *et al* 1980; Levin *et al* 1984; Casonato *et al* 1992; Tranquille *et al* 1992]. One can, therefore, only speculate as to whether our findings extend to the acute t-PA release seen with *in situ* thrombosis. However, both nitric oxide mediated endothelial dysfunction [Panza *et al* 1994; Casino *et al* 1995; Stroes *et al* 1995; Celermajer *et al* 1996] and abnormalities of endogenous fibrinolysis [Hamsten *et al* 1985; Ridker *et al* 1993a; Ridker *et al* 1994; Thompson *et al* 1995] have been described in many atherosclerotic diseases and the associated risk factors. Thus, the coupling of acute t-PA release to the L-arginine:nitric oxide pathway provides an important potential mechanism whereby endothelial dysfunction might increase the risk of atherothrombosis through a reduction in the acute fibrinolytic capacity. Our initial findings would suggest that this model could be applied to the assessment of the acute fibrinolytic capacity of patients with endothelial dysfunction such as those with hypercholesterolaemia and a smoking habit [Stroes *et al* 1995; Celermajer *et al* 1996], and to the examination of the subsequent effect of L-arginine supplementation.

Substance P induced vasodilatation undergoes tachyphylaxis [McEwan *et al* 1988] which may relate to internalisation of the neurokinin type 1 receptor from the endothelial cell surface membrane [Bowden *et al* 1994]. It has been suggested from *ex vivo* animal studies [Kuroiwa *et al* 1995; Gross *et al* 1994] that the residual vasodilatation following the development of tachyphylaxis is almost completely nitric oxide dependent. In the present study, the degree of inhibition of substance P induced vasodilatation by L-NMMA was less than we [Newby *et al* 1997c] and others [Cockcroft *et al* 1994] have previously described and may reflect the higher

potency and doses used in this study. Whilst we have readily demonstrated tachyphylaxis of substance P induced vasodilatation, the co-infusion of L-NMMA did not affect the development of tachyphylaxis and did not abolish the residual substance P induced vasodilatation following its development. Thus, in contrast to animal studies, residual vasodilatation after the development of tachyphylaxis does not appear to be predominantly nitric oxide mediated in the human forearm. In addition, we were unable to detect significant tachyphylaxis of substance P induced increases in plasma t-PA antigen and activity concentrations suggesting that not all the actions of substance P undergo tachyphylaxis.

The substance P induced reductions in plasma PAI-1 activity of the infused arm without significant alterations in PAI-1 antigen concentrations are consistent with acute t-PA release in the absence of PAI-1 release [Chandler et a 1997]. PAI-1 binds to the newly released t-PA to form an inactive PAI-1/t-PA complex, thereby reducing the plasma PAI-1 activity. The trend for PAI-1 antigen concentrations to fall in both arms as the study progressed is consistent with systemic (hepatic) clearance of the PAI-1/t-PA complex [Lucore *et al* 1988; Otter *et al* 1992; Chandler *et al* 1997]. However, this trend was also seen with isolated L-NMMA infusion in which there was no significant release of t-PA consistent with a circadian fall of PAI-1 antigen during the morning [Andreotti *et al* 1991].

Despite reducing forearm blood flow by half, L-NMMA did not significantly affect the constitutive release or plasma concentrations of t-PA and PAI-1 antigen and activity. The 95% confidence intervals indicates that if L-NMMA has an effect on

basal t-PA or PAI-1 release then it is rather small. This suggests that the L-arginine:nitric oxide pathway does not play a major role in the basal release of t-PA or PAI-1 in the peripheral vasculature of man.

7.5.1 Study limitations

Since the derivation of t-PA release is a function of plasma flow, it could be argued that the inhibition by L-NMMA of substance P induced t-PA release reflects the simultaneous reduction in blood flow. However, the reduction in absolute blood flow was only modest (15 to 20%) in comparison to the reduction in t-PA release (40 to 46%) and the plasma t-PA activity concentrations in the infused forearm were also significantly reduced by co-infusion of L-NMMA. The findings of the present study would be strengthened by utilising a control vasoconstrictor and demonstrating a neutral effect on substance P induced t-PA release. However, standard receptor coupled vasoconstrictors used in forearm studies, such as noradrenaline, vasopressin and angiotensin II, are known to stimulate t-PA and PAI-1 release [Ludlam *et al* 1980; Casonato *et al* 1992; Ridker *et al* 1993; Jern *et al* 1997a; Wall *et al* 1998; Larsson et al 1999] and would not help in interpreting the influence of L-NMMA on substance P induced t-PA release.

We have previously been unable to detect an acute local release of either vWf or PAI-1 during 10 minute infusions of substance P given at 8-fold higher concentrations [Newby *et al* 1997b]. In the present study, substance P did not cause significant vWf or PAI-1 release despite infusion times of up to 120 minutes suggesting that the dissociation of substance P induced t-PA release from vWf is not

a temporal effect. However, this dissociated release does not appear to be unique to substance P since this has also been recently described with local forearm infusions of desmopressin [Wall *et al* 1998]. These findings are, however, limited to the peripheral forearm vascular bed and the endothelium in other tissue beds may respond differently to substance P stimulation. The extension of this model to vascular beds associated with atherosclerosis such as the coronary circulation, will be of crucial relevance in determining the influence of atheroma and endothelial dysfunction on the acute local release of t-PA during thrombotic occlusion and plaque rupture.

In summary, in the forearm vascular bed *in vivo*, we have shown for the first time that the L-arginine:nitric oxide pathway contributes to substance P induced t-PA release in man. This coupling of acute t-PA release to the L-arginine:nitric oxide pathway provides an important potential mechanism whereby endothelial dysfunction increases the risk of atherothrombosis through a reduction in the acute fibrinolytic capacity.

CHAPTER 8

ENDOTHELIAL DYSFUNCTION, IMPAIRED ENDOGENOUS FIBRINOLYSIS AND CIGARETTE SMOKING: A MECHANISM FOR ARTERIAL THROMBOSIS AND MYOCARDIAL INFARCTION

Newby DE, Wright RA, Labinjoh C, Ludlam CA, Fox KAA, Boon NA, Webb DJ. Endothelial dysfunction, impaired endogenous fibrinolysis and cigarette smoking: a mechanism for arterial thrombosis and myocardial infarction. *Circulation* 1999;**99**:1411-1415.

8.1 SUMMARY

Effective endogenous fibrinolysis requires rapid release of tissue plasminogen activator (t-PA) from the vascular endothelium. Smoking is a known risk factor for arterial thrombosis and myocardial infarction, and causes endothelial dysfunction. We, therefore, examined the effects of cigarette smoking on substance P induced t-PA release *in vivo* in man. Blood flow and plasma fibrinolytic factors were measured in both forearms of 12 smokers and 12 age- and sex-matched non-smokers who received unilateral brachial artery infusions of substance P (2 to 8 pmol/min). In both smokers and non-smokers, substance P caused dose-dependent increases in blood flow and local release of plasma t-PA antigen and activity ($p<0.001$ for all) but had no effect on the local release of plasminogen activator inhibitor type 1. In comparison to non-smokers, increases in forearm blood flow ($p=0.03$), and release of t-PA antigen ($p=0.04$) and activity ($p<0.001$) caused by substance P, were reduced in smokers. Area under the curve for release of t-PA antigen and activity decreased by 51% and 53% respectively. Cigarette smoking causes marked inhibition of substance P induced t-PA release *in vivo* in man. This provides an important mechanism whereby endothelial dysfunction may increase the risk of atherothrombosis through a reduction in the acute fibrinolytic capacity.

8.2 INTRODUCTION

Acute rupture or erosion of a coronary atheromatous plaque and subsequent coronary artery thrombosis causes the majority of sudden cardiac deaths and myocardial infarctions [Burke *et al* 1997; Davies 1997]. Cigarette smoking is not only strongly associated with atherosclerosis [Chen *et al* 1995] and ischaemic heart disease [Njolstad *et al* 1996], but is also a major risk factor for acute coronary thrombosis [Hung *et al* 1995; Burke *et al* 1997]. Indeed, three-quarters of sudden cardiac deaths due to acute thrombosis are in cigarette smokers [Burke *et al* 1997]. Smoking causes endothelial dysfunction [Celermajer *et al* 1996] and is associated with increased platelet thrombus formation [Hung *et al* 1995]. Small areas of denudation and thrombus deposition are a common finding on the surface of atheromatous plaques [Davies *et al* 1988; Bürrig 1991] and are usually sub-clinical. However, in the presence of an imbalance in the coagulation or fibrinolytic systems, such microthrombi may propagate, ultimately leading to arterial occlusion.

Using the endothelium-dependent vasodilator, substance P, to stimulate t-PA release we have recently described an *in vivo* model to assess the acute fibrinolytic capacity of the human forearm [Newby *et al* 1997b]. Moreover, we have been able to demonstrate a reduction in t-PA release after inducing experimental 'endothelial dysfunction' with nitric oxide synthase inhibition [Newby *et al* 1998]. We, therefore, hypothesised that cigarette smoking might impair endogenous fibrinolysis by reducing the capacity of the endothelium to release t-PA acutely. The aim of the

study was to compare substance P induced t-PA release from the forearm vascular bed of smokers and age- and sex-matched non-smokers.

8.3 METHODS

8.3.1 Subjects

Twelve healthy smokers (5 to 20 cigarettes/day), and 12 age- and sex-matched non-smokers, aged between 25 and 55 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.

All subjects were normotensive without a history of diabetes mellitus or vascular disease. Female subjects were premenopausal and not receiving hormonal contraceptives. They were clinically well and taking no regular medications. Control subjects were life-long 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 standing and maintained their normal smoking habit in the week before attendance.

8.3.2 Drugs

Pharmaceutical-grade substance P (Clinalfa AG, Läufelfingen, Switzerland) was administered following dissolution in saline.

8.3.3 Study Design

At 9.00 am, subjects attended fasted and then rested recumbent throughout each study. Strain gauges and cuffs were applied and the brachial artery of the non-dominant arm cannulated. Forearm blood flow was measured every 10 minutes.

Saline was infused for the first 30 minutes to allow time for equilibration. The final blood flow measurement during saline infusion was taken as the basal forearm blood flow. Thereafter, subjects received intra-arterial substance P at 2, 4 and 8 pmol/min, for 10 minutes at each dose.

8.3.4 Data Analysis And Statistics

The study population size, based on power calculations derived from previous studies [Newby *et al* 1997b], gives 90% power of detecting a 18% difference in t-PA release at a significance level of 5%. Coefficients of repeatability [Bland & Altman 1986] for plasma concentrations of t-PA antigen and activity during substance P infusion at 8 pmol/min are 1.6 ng/mL and 1.4 IU/mL respectively [Newby *et al* 1999b].

Data were examined, where appropriate, by two-way analysis of variance (ANOVA) with repeated measures and two tailed Student's *t*-test using Excel *v*5.0 (Microsoft). Area under the curve (AUC) was calculated for the estimated net release of t-PA across the study period. All results are expressed as mean ± standard error of the mean. Statistical significance was taken at the 5% level.

8.4 RESULTS

There were no significant differences in baseline characteristics except smokers had a slightly lower high density lipoprotein concentration (Table 8.1). There were no significant changes in blood pressure, heart rate, haematocrit or blood flow in the non-infused forearm during the study (Table 8.2). In the non-infused arm, plasma t-PA antigen concentrations were higher in smokers than non-smokers ($p=0.02$; Table 8.2). There were no significant differences in plasma PAI-1 antigen and activity between the groups.

Substance P caused dose-dependent increases in forearm blood flow in the infused arm in both smokers and non-smokers (Table 8.2, Figure 8.1) but the increase in blood flow was greater in non-smokers ($p=0.03$; two-way ANOVA: non-smokers *vs* smokers). In comparison to the non-infused arm (two-way ANOVA), substance P caused dose-dependent increases in plasma concentrations of t-PA antigen ($p<0.001$) and activity ($p<0.001$) in the infused arm of both smokers and non-smokers (Table 8.2). There were no significant changes in plasma PAI-1 antigen or activity in either group. The increase in plasma t-PA activity in the infused arm was greater in the non-smokers ($p=0.001$; two-way ANOVA: non-smokers *vs* smokers).

Substance P increased the net release of t-PA antigen ($p=0.009$) and activity ($p<0.001$) in smokers (Figure 8.1). In non-smokers, substance P increased the net release of t-PA antigen ($p<0.001$) and activity ($p<0.001$) significantly more than in smokers ($p=0.04$ and $p<0.001$ respectively; two-way ANOVA: non-smokers *vs*

Table 8.1.
Baseline subject characteristics

	Non-Smokers	Smokers	
Age (years)	35 ± 3	34 ± 2	
Sex (male:female)	10 : 2	10 : 2	
Body Mass Index (kg/m^2)	23.9 ± 0.5	24.5 ± 1.1	
Mean Arterial Pressure (mmHg)	92 ± 2	91 ± 2	
Heart Rate (/min)	59 ± 3	66 ± 2	
Fasting Plasma Glucose (mmol/L)	5.3 ± 0.1	5.4 ± 0.1	
Total Cholesterol (mmol/L)	5.0 ± 0.4	5.3 ± 0.4	
LDL Cholesterol (mmol/L)	3.0 ± 0.3	3.4 ± 0.4	
HDL Cholesterol (mmol/L)	1.3 ± 0.1	1.0 ± 0.1	*
Triglycerides (mmol/L)	1.6 ± 0.2	1.9 ± 0.3	
Baseline Haematocrit	0.41 ± 0.01	0.43 ± 0.01	

*p = 0.01 (unpaired *t*-test, smokers *vs* non-smokers)

Table 8.2.
Blood flow and plasma tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) antigen and activity concentrations in both forearms

		Non-smokers					Smokers				
Time	(min)	**Baseline**	**10**	**20**	**30**		**Baseline**	**10**	**20**	**30**	
Substance P Dose	(pmol/min)	**0**	**2**	**4**	**8**		**0**	**2**	**4**	**8**	
Absolute Forearm Blood Flow (mL/100 mL/min)	**Non-infused Arm**	2.8 ± 0.3	2.9 ± 0.4	2.9 ± 0.4	2.8 ± 0.3		2.8 ± 0.2	2.9 ± 0.3	2.9 ± 0.3	3.0 ± 0.3	
	Infused Arm	3.7 ± 0.4	11.2 ± 1.1	13.5 ± 1.3	16.2 ± 1.5	*	3.6 ± 0.3	9.4 ± 0.4	11.5 ± 0.7	14.2 ± 0.8	* †
t-PA Antigen (ng/mL)	**Non-infused Arm**	3.3 ± 0.5	3.4 ± 0.5	3.4 ± 0.5	3.7 ± 0.5		4.0 ± 0.5	4.3 ± 0.5	4.4 ± 0.5	4.4 ± 0.6	†
	Infused Arm	3.2 ± 0.5	4.1 ± 0.6	4.4 ± 0.6	6.2 ± 0.8	*	4.1 ± 0.5	4.5 ± 0.6	5.2 ± 0.7	5.9 ± 0.9	*
t-PA Activity (IU/mL)	**Non-infused Arm**	0.8 ± 0.2	0.9 ± 0.2	1.0 ± 0.2	1.3 ± 0.2		0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	1.0 ± 0.2	
	Infused Arm	0.8 ± 0.2	2.1 ± 0.5	2.8 ± 0.5	4.6 ± 0.6	*	0.7 ± 0.1	1.1 ± 0.2	1.7 ± 0.4	3.0 ± 0.5	* ‡
PAI-1 Antigen (ng/mL)	**Non-infused Arm**	29 ± 7	29 ± 6	28 ± 7	28 ± 6		29 ± 6	26 ± 5	25 ± 5	26 ± 5	
	Infused Arm	28 ± 6	28 ± 7	27 ± 6	28 ± 5		26 ± 5	27 ± 6	26 ± 6	26 ± 5	
PAI-1 Activity (AU/mL)	**Non-infused Arm**	11.8 ± 1.7	11.8 ± 1.7	12.1 ± 1.6	11.4 ± 1.8		12.0 ± 2.0	11.0 ± 1.7	9.2 ± 1.4	10.2 ± 1.3	
	Infused Arm	10.7 ± 1.6	8.8 ± 1.5	10.8 ± 1.7	9.3 ± 1.5		12.5 ± 1.9	10.6 ± 1.5	10.5 ± 1.2	8.5 ± 1.1	

One-way ANOVA: * $p < 0.001$
Two-way ANOVA (non-smokers *vs* smokers): † $p < 0.05$; ‡ $p = 0.001$

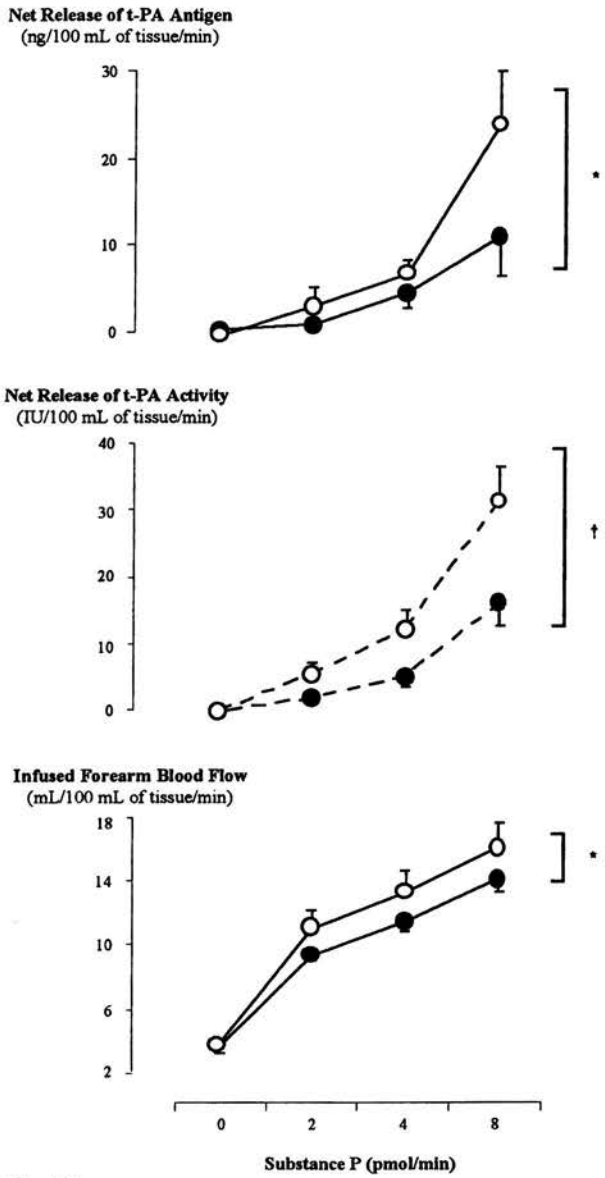


Figure 8.1.
Infused forearm blood flow and the net release of t-PA antigen and activity in smokers (solid circles) and non-smokers (open circles).
One-way ANOVA: $p<0.001$ for all responses.
Two way ANOVA (non-smokers *vs* smokers): * $p<0.05$; † $p<0.001$

smokers). In comparison to the non-smokers, the AUC for net t-PA antigen and activity release was reduced by 51% and 53% respectively in the smokers.

Subgroup analysis following exclusion of female subjects did not alter the magnitude or the statistical significance of the above findings. Qualitatively, the responses in female smokers and non-smokers were similar to those observed in the male subjects.

8.5 DISCUSSION

We have shown here, for the first time, that despite higher basal plasma t-PA antigen concentrations, cigarette smokers have a markedly impaired capacity of the endothelium to release t-PA acutely. This establishes an important mechanism whereby cigarette smoking can lead to arterial thrombosis and myocardial infarction.

The rapid mobilisation of t-PA from the endothelium is crucial if endogenous fibrinolysis within the arterial circulation is to be effective, with thrombus dissolution being much more effective if t-PA is incorporated during, rather than after, thrombus formation [Brommer 1984 Fox *et al* 1984]. The increased risk of spontaneous thrombosis seen in smokers may, therefore, plausibly relate to the propagation of thrombus which would otherwise undergo lysis and remain sub-clinical. Although cigarette smokers have a higher overall mortality from myocardial infarction than non-smokers [Håheim *et al* 1993], the in-hospital mortality is lower [Mueller *et al* 1992; Barbash *et al* 1993; Zahger *et al* 1995]. This apparent paradox can be explained by the observation that the infarct related artery is more than twice as likely to become patent in current smokers compared to non-smokers following thrombolytic therapy for acute myocardial infarction [Gomez *et al* 1993; Zahger *et al* 1995; De Chillou *et al* 1996]. Indeed, it has been suggested [De Chillou *et al* 1996; Bowers *et al* 1996] that thrombolytic therapy should only be given to smokers and alternative strategies such as primary angioplasty used in non-smokers. These observations are consistent with the current findings since it might be anticipated that patients with impaired endothelial cell t-PA release would benefit most from

thrombolytic therapy whilst those with a normal endogenous fibrinolytic capacity are more likely to have t-PA resistant thrombus which would respond less favourably.

Our findings in smokers are consistent with the previous observational data [Hamsten *et al* 1985; Ridker *et al* 1993a; Thompson *et al* 1995] that increased basal plasma concentrations of t-PA antigen are associated with future coronary events. The assessment of endogenous fibrinolysis has previously relied on measurement of basal plasma t-PA concentrations and the acute release of t-PA in response to venous occlusion, systemic desmopressin infusion or exercise [Allen *et al* 1985; Gris *et al* 1991; Jahun-Vague *et al* 1996; Held *et al* 1997]. However, because of confounding systemic effects and the non-uniformity of the stimuli applied, these responses can be variable and give only a relatively crude measure of fibrinolytic capacity. Moreover, although it has previously been shown that systemic desmopressin infusion causes less t-PA release in smokers [Allen *et al* 1985], this effect may not be directly endothelium dependent [Mannucci 1997]. In contrast, we have used locally active doses of substance P to provide a more precise pharmacological stimulus to the endothelium, and to cause a substantial and dose-dependent local release of t-PA [Newby *et al* 1997b; Newby *et al* 1998]. This has enabled us to demonstrate a distinct and marked inhibition of stimulated endothelial t-PA release in smokers.

Although thrombin is more physiologically relevant to acute t-PA release than substance P, we have used the latter because its vascular actions are endothelium-dependent [Gross *et al* 1994], mediated in part through nitric oxide [Newby *et al* 1997c], and its administration intra-arterially is safe and well tolerated [Newby *et al*

1997a]. Consistent with previous workers [Celermajer *et al* 1996; Heitzer *et al* 1996a; Heitzer *et al* 1996b], we have also found an attenuation of the endothelium-dependent forearm blood flow responses in smokers. This inhibition of both the blood flow and t-PA response may, in part, relate to an impairment of the L-arginine:nitric oxide pathway in smokers [Heitzer *et al* 1996a; Newby *et al* 1998]. Whilst differences exist [Hirooka *et al* 1994], the forearm model may provide a useful surrogate for the coronary vascular bed [Sax *et al* 1987; Pedrinelli *et al* 1993] and permits a readily accessible and reliable assessment of endothelial cell function. However, the present findings need to be confirmed in the coronary circulation.

We have studied the sustained effect of chronic smoking in a selected healthy and predominantly male population at a single time point. Although total and low density lipoprotein cholesterol concentrations were similar in both smokers and non-smokers, high density lipoprotein cholesterol concentrations were slightly lower in smokers. This is not unexpected since cigarette smoking is known to be associated with a selective reduction in HDL cholesterol concentrations [Shennan *et al* 1985; Ferrara *et al* 1997]. However, the application of this model to other conditions associated with endothelial dysfunction, such as dyslipidaemia, is warranted. Finally, since hormonal status influences fibrinolytic parameters [Koh *et al* 1997], the assessment of the acute fibrinolytic capacity in pre and postmenopausal women, and the modulating effect of hormonal therapy, will also be of particular interest.

In conclusion, we have demonstrated a major impairment of t-PA release from the vascular endothelium of smokers. Our findings suggest that the fundamental

mechanism whereby cigarette smoking causes arterial thrombosis and myocardial infarction relates, at least in part, to impairment of the acute endogenous fibrinolytic capacity.

CHAPTER 9

HYPERCHOLESTEROLAEMIA AND LIPID LOWERING THERAPY DO NOT AFFECT THE ACUTE ENDOGENOUS FIBRINOLYTIC CAPACITY *IN VIVO*

Newby DE, Witherow FN, Wright RA, Bloomfield P, Ludlam CA, Boon NA, Fox KAA, Webb DJ.
Hypercholesterolemia and lipid lowering therapy do not affect the acute endogenous fibrinolytic capacity *in vivo*.
Under submission.

9.1 SUMMARY

Endothelial dysfunction, demonstrated by impaired endothelium-dependent vasodilatation, is found in patients with hypercholesterolaemia. The aims of the present study were to assess acute tissue plasminogen activator (t-PA) release *in vivo* in patients with hypercholesterolaemia, in the presence and absence of lipid lowering therapy, and in matched normocholesterolaemic controls. Blood flow and plasma fibrinolytic factors were measured in both forearms of eight patients with hypercholesterolaemia (>7.8 mmol/L) and eight matched normocholesterolaemic controls (<5.5 mmol/L). Subjects received unilateral brachial artery infusions of the endothelium-dependent vasodilator, substance P (2 to 8 pmol/min), and the endothelium-independent vasodilator, sodium nitroprusside (1 to 4 μg/min). In patients, measurements were made on 3 occasions: at baseline and after 6 weeks of placebo or pravastatin 40 mg daily administered in a double blind, randomised, crossover design. In comparison to patients, substance P caused greater dose-dependent increases in forearm blood flow ($p<0.05$) in normocholesterolaemic controls, but similar increases in plasma t-PA antigen and activity concentrations. During pravastatin therapy in patients, total serum cholesterol fell by 22% from 8.1 ± 0.3 to 6.4 ± 0.4 mmol/L ($p=0.002$) and substance P induced vasodilatation was no longer significantly impaired in comparison to controls. However, despite reproducible responses, pravastatin therapy was not associated with significant changes in basal or substance P induced t-PA release. Hypercholesterolaemia and lipid lowering therapy cause no demonstrable effects on acute substance P induced t-

PA release *in vivo*. This suggests that the preventative benefits of lipid lowering therapy are unlikely to be mediated by improvements in endogenous fibrinolysis.

9.2 INTRODUCTION

Hypercholesterolaemia impairs endothelial cell function [Creager *et al* 1990; Chowienczyk *et al* 1992; Casino *et al* 1995], predisposes to vessel damage and contributes to vascular occlusion [Burke *et al* 1997]. Previous studies have shown that endothelium-dependent, nitric oxide mediated vasodilatation is impaired in patients with hypercholesterolaemia [Creager *et al* 1990; Chowienczyk *et al* 1992; Casino *et al* 1995], an effect which is reversed by lipid lowering therapy [Egashira *et al* 1994; Stroes *et al* 1995; O'Driscoll *et al* 1997]. Further measures of endothelial cell dysfunction are lacking but the fibrinolytic factor, t-PA and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1), are potentially important markers and are intimately linked to the risk of atherothrombosis [Hamsten *et al* 1985;Thompson *et al* 1995].

We have recently described an *in vivo* model to assess the acute release of t-PA in the forearm of man [Newby *et al* 1997b]. Using intra-brachial infusions of substance P, we have shown a dose-dependent release of t-PA without causing a significant release in PAI-1. Moreover, we have also reported [Newby *et al* 1998] that t-PA release is inhibited by nitric oxide synthase inhibition with L-N^G-monomethylarginine suggesting that endothelial dysfunction may impair the release of t-PA. Given that substance P induced vasodilatation has been reported to be impaired in patients with hypercholesterolemia [Casino *et al* 1995], the aims of the present study were to determine whether there is also an impairment of t-PA release

in patients with hypercholesterolaemia, and if treatment with pravastatin [Egashira *et* al 1994] could enhance t-PA release in these patients.

9.3 METHODS

9.3.1 Patients And Control Subjects

Patients with primary hypercholesterolaemia were recruited from the clinic if their serum cholesterol concentrations exceeded 7.8 mmol/L. Following screening with clinical examination, repeated questioning for symptoms, clinical chemistry screen (liver enzymes, electrolytes, urea and creatinine), haematology screen (full blood and differential count), urinalysis and 12-lead electrocardiogram, patients were excluded if they had diabetes mellitus, hypertension, ischaemic heart disease, peripheral vascular disease, an abnormal resting electrocardiogram or other clinically significant disease. Patients were matched with normocholesterolaemic (<5.5 mmol/L) healthy controls for age, sex and smoking habit. All studies were undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki.

9.3.2 Drugs

Pharmaceutical-grade substance P (Clinalfa AG, Läufelfingen, Switzerland) and sodium nitroprusside (David Bull Laboratories, Warwick, U.K.) were administered following dissolution in saline. Pravastatin (Bristol-Myers Squibb, Hounslow, U.K.) 40 mg daily or sucrose placebo capsules were administered orally.

9.3.3 Serum Lipid Assays

Serum cholesterol and triglyceride concentrations were determined by an enzymatic colorimetric method (Boehringer Mannheim GmbH Diagnostica, Mannheim,

Germany). LDL cholesterol was determined by the method of Friedewald and colleagues [Freidewald *et al* 1972].

9.3.4 Study Design

All patients attended on each of the three separate study days: baseline and following 6 weeks treatment with placebo and 6 weeks with pravastatin 40 mg daily. Placebo and pravastatin treatments were given in a randomised, double blind, crossover design. Control subjects attended on one occasion only.

On each study day, subjects attended fasted at 09.00 hours and rested recumbent throughout. Strain gauges and cuffs were applied and the brachial artery of the non-dominant arm cannulated. Throughout all protocols, measurements of forearm blood flow were made every 10 minutes. Before substance P and sodium nitroprusside administration, saline was infused for 30 minutes to allow time for equilibration and the final blood flow measurement during saline infusion was taken as the basal forearm blood flow. Substance P was infused at 2, 4 and 8 pmol/min for 10 minutes at each dose [Newby *et al* 1997b; Newby *et al* 1998; Newby *et al* 1999a] and sodium nitroprusside was infused at 1, 2 and 4 μg/min for 10 minutes at each dose [Newby *et al* 1997b]. The order of substance P and sodium nitroprusside was randomised.

9.3.5 Data Analysis And Statistics

Data were examined, where appropriate, by two-way analysis of variance (ANOVA) with repeated measures and two tailed paired Student's *t*-test using Excel *v*5.0 (Microsoft). Reproducibility of the responses to substance P infusion was assessed

by comparing the baseline and placebo treatment study days using the method of Bland and Altman [Bland & Altman 1986] Coefficients of reproducibility were determined for 95% confidence intervals using the Student's *t* distribution. All results are expressed as mean ± standard error of the mean. Statistical significance was taken at the 5% level. Based on previous data [Newby *et al* 1997b] the study had 90% power to detect a 20% change in plasma t-PA concentrations between treatment periods at the 5% level.

9.4 RESULTS

Patient and control subject characteristics are shown in Table 9.1. The groups were well matched for age, sex, body mass index, smoking habit, blood pressure, heart rate, haematocrit and forearm blood flow. Total cholesterol and LDL cholesterol concentrations were significantly higher in the patient group and fell during pravastatin but not placebo treatment (Table 9.1). On each of the study days in the patient and control groups, blood pressure, heart rate, haematocrit, and the blood flow and t-PA concentrations of the non-infused forearm did not change.

9.4.1 Blood Flow Responses

Substance P caused dose dependent increases in blood flow of the infused forearm in both patients and controls ($p<0.001$ for both; ANOVA: Figure 9.1). In comparison to the control group, the substance P induced increases in blood flow were significantly less than in the patient group ($p<0.05$; two-way ANOVA: Figure 9.1). During pravastatin treatment, substance P appeared to cause a slightly greater increase in blood flow but this was not statistically significant from baseline responses ($p=0.30$; two-way ANOVA, baseline *vs* pravastatin) although it was no longer significantly different from the control group ($p=0.24$; two-way ANOVA).

Sodium nitroprusside also caused dose-dependent increases in blood flow of the infused forearm ($p<0.001$; ANOVA: Figure 9.1) which were similar in patient and control groups. Neither pravastatin nor placebo therapy influenced the response to sodium nitroprusside infusion.

Table 9.1.
Lipid profile, haemodynamics, absolute forearm blood flow and haematocrit at baseline, and during placebo and pravastatin treatment

	Controls	Patients			
		Baseline	Placebo	Pravastatin	
Age	43 ± 3	46 ± 3	-	-	(years)
Sex	5:3	5:3	-	-	(male:female)
Body Mass Index	24 ± 1	25 ± 1	-	-	(kg/m^2)
Smokers	3	3	-	-	
Total cholesterol	5.0 ± 0.2*	8.1 ± 0.3	8.2 ± 0.3	6.4 ± 0.4†	(mmol/L)
LDL cholesterol	3.2 ± 0.2*	5.8 ± 0.5	6.1 ± 0.5	4.3 ± 0.4‡	(mmol/L)
HDL cholesterol	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	1.2 ± 0.2	(mmol/L)
Triglycerides	1.5 ± 0.8	2.7 ± 1.0	2.3 ± 0.8	1.9 ± 0.3	(mmol/L)
Mean arterial pressure	88 ± 5	87 ± 2	87 ± 4	87 ± 2	(mmHg)
Heart rate	64 ± 2	62 ± 3	63 ± 5	62 ± 4	(/min)
Basal blood flow:					
Non-infused arm	3.0 ± 0.5	2.4 ± 0.3	2.9 ± 0.3	2.4 ± 0.3	(mL/100 mL/min)
Infused arm	3.7 ± 0.7	3.6 ± 0.3	3.2 ± 0.3	2.5 ± 0.3	(mL/100 mL/min)
Haematocrit	0.39 ± 0.01	0.43 ± 0.01	0.42 ± 0.01	0.41 ± 0.01	

Unpaired *t*-test *p<0.001 (controls *vs* patients - baseline)
†p=0.003; ‡p=0.04 (patients - pravastatin *vs* controls)
Paired *t*-test †p<0.003; ‡p=0.02 (patients - placebo *vs* pravastatin)
ANOVA †p<0.002; ‡p=0.04 (three-way ANOVA for patient groups)

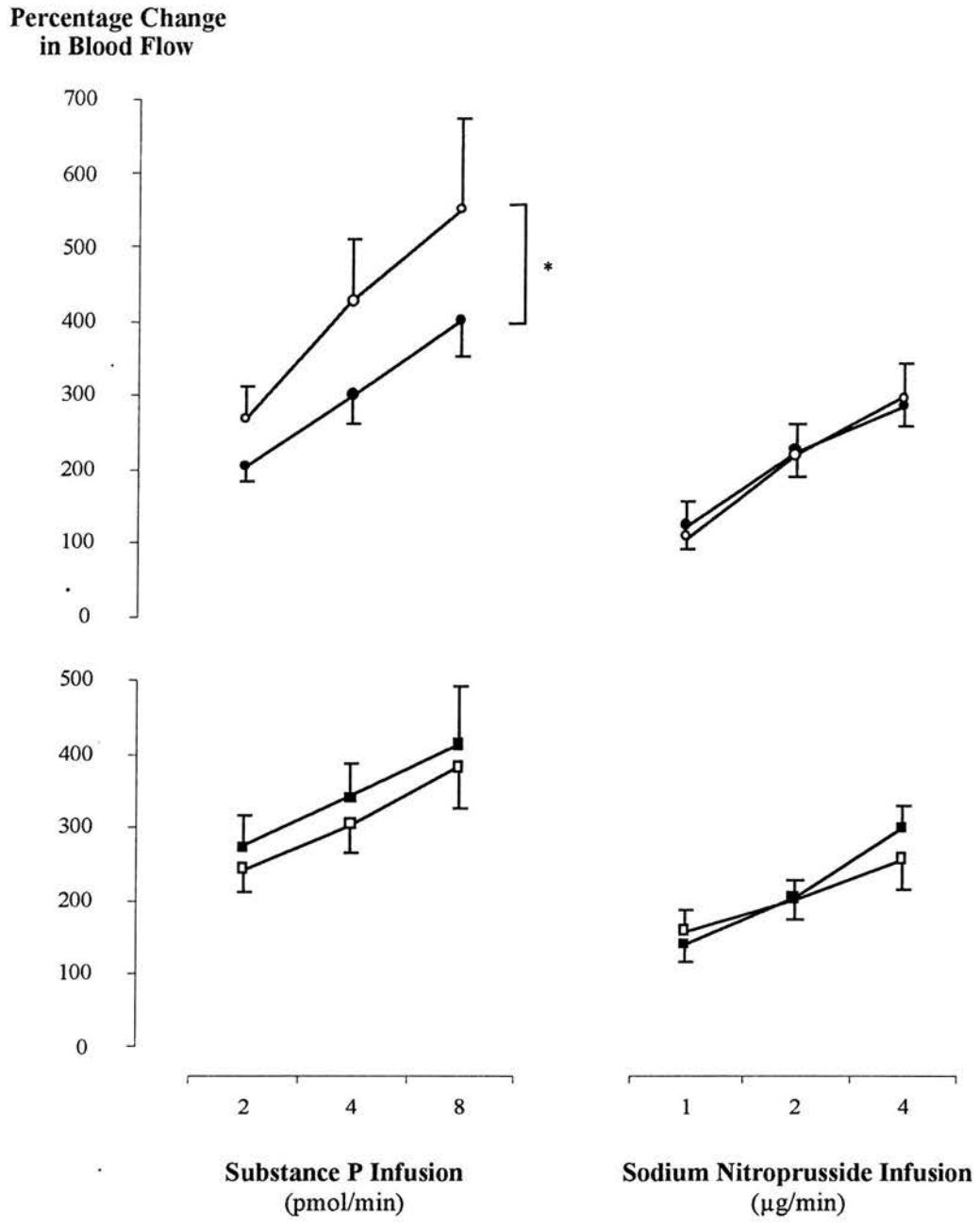


Figure 9.1
Forearm blood flow responses to substance P and sodium nitroprusside administration in controls (open circles; upper panels) and patients at baseline (closed circles; upper panels), and in patients during placebo (open squares; lower panels) and during pravastatin (closed squares; lower panels).
$p<0.001$ for each forearm blood flow response (one-way ANOVA)
*$p<0.05$ (2-way ANOVA, controls *vs* patients)

9.4.2 Fibrinolytic Factor Responses

Substance P caused dose-dependent increases in plasma t-PA antigen and activity concentrations in the infused forearm of both patients and controls ($p<0.001$ for both; ANOVA: Table 9.2). Substance P induced increases in plasma t-PA concentrations were similar in both groups and, in the patient group, was unaffected by pravastatin or placebo therapy. The concentration differences between the forearms and the estimated net release of t-PA antigen and activity also demonstrated dose-dependent increases ($p<0.001$; ANOVA: Figure 9.2) which did not differ between the patient and control groups or during pravastatin and placebo therapy. There were no significant differences in plasma PAI-1 antigen and activity concentrations at baseline, although there appeared to be a trend for plasma PAI-1 antigen concentrations to be lower in the control group ($p>0.12$; unpaired *t*-test: Table 9.3). Plasma PAI-1 concentrations were unaffected by substance P infusion or pravastatin treatment (Table 9.3).

9.4.3 Reproducibility Of Substance P Responses

Comparison of responses at baseline and during placebo therapy demonstrates good reproducibility for substance P induced increases in plasma t-PA concentrations and forearm blood flow (Table 9.4).

Table 9.2
Plasma t-PA antigen and activity in patients at baseline, and during placebo and pravastatin treatment, and in a matched control group

		Controls	Patients		
	Substance P (pmol/min)		**Baseline**	**Placebo**	**Pravastatin**
t-PA Antigen (ng/mL)					
Infused Forearm*	**0**	5.0 ± 0.5	4.6 ± 0.8	4.7 ± 0.6	4.5 ± 0.6
	2	5.6 ± 0.7	5.4 ± 0.8	5.4 ± 0.6	5.0 ± 0.4
	4	7.4 ± 0.8	5.7 ± 0.9	6.0 ± 0.9	5.8 ± 0.6
	8	8.0 ± 1.3	7.4 ± 1.1	7.5 ± 1.3	7.6 ± 1.1
Non-infused Forearm	**0**	4.7 ± 0.5	4.8 ± 0.7	4.5 ± 0.6	4.6 ± 0.6
	2	4.6 ± 0.4	4.8 ± 0.7	4.5 ± 0.5	4.5 ± 0.6
	4	5.8 ± 0.6	5.1 ± 0.8	4.5 ± 0.8	4.6 ± 0.6
	8	5.7 ± 0.6	5.0 ± 0.8	5.1 ± 0.6	4.8 ± 0.6
t-PA Activity (IU/mL)					
Infused Forearm*	**0**	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
	2	1.7 ± 0.5	1.3 ± 0.5	1.1 ± 0.4	1.6 ± 0.8
	4	2.7 ± 1.0	2.0 ± 0.6	1.7 ± 0.9	2.2 ± 1.1
	8	4.5 ± 1.5	3.4 ± 1.1	3.9 ± 1.4	3.1 ± 1.2
Non-infused Forearm	**0**	0.7 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
	2	0.8 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	0.5 ± 0.1
	4	1.1 ± 0.2	0.6 ± 0.1	0.7 ± 0.2	0.6 ± 0.1
	8	1.7 ± 0.5	0.7 ± 0.2	0.8 ± 0.2	0.7 ± 0.2

*$p<0.001$, one-way ANOVA in the infused forearm for each group

Table 9.3
Plasma PAI-1 antigen and activity in patients at baseline, and during placebo and pravastatin treatment, and in matched controls

		Controls	Patients		
	Substance P (pmol/min)		**Baseline**	**Placebo**	**Pravastatin**
PAI-1 antigen (ng/mL)					
Infused arm	**0**	23 ± 9	40 ± 10	47 ± 12	48 ± 15
	2	24 ± 8	43 ± 10	47 ± 11	44 ± 13
	4	24 ± 8	41 ± 9	46 ± 11	51 ± 16
	8	29 ± 10	39 ± 9	46 ± 12	46 ± 14
Non-infused arm	**0**	22 ± 8	44 ± 11	50 ± 13	50 ± 16
	2	23 ± 8	40 ± 9	51 ± 12	51 ± 18
	4	26 ± 9	39 ± 9	51 ± 14	46 ± 14
	8	28 ± 8	38 ± 9	48 ± 11	46 ± 14
PAI-1 activity (AU/mL)					
Infused arm	**0**	9 ± 3	13 ± 2	15 ± 3	13 ± 3
	2	10 ± 4	10 ± 2	16 ± 3	13 ± 2
	4	9 ± 4	9 ± 3	13 ± 3	14 ± 2
	8	11 ± 5	11 ± 3	13 ± 3	13 ± 2
Non-infused arm	**0**	12 ± 3	14 ± 3	14 ± 3	15 ± 3
	2	8 ± 4	13 ± 2	16 ± 3	13 ± 2
	4	11 ± 5	10 ± 3	14 ± 2	13 ± 3
	8	9 ± 4	12 ± 3	13 ± 3	12 ± 3

Table 9.4
Repeatability of t-PA and blood flow responses to substance P administration in the infused forearm: baseline *vs* placebo

	Substance P (pmol/min)	Baseline Mean	Placebo Mean	Mean of Differences	Coefficient of Repeatability
t-PA antigen (ng/mL)	**0**	4.6 ± 0.8	4.7 ± 0.6	0.5	0.4
	2	5.4 ± 0.8	5.4 ± 0.6	0.1	1.4
	4	5.7 ± 0.9	6.0 ± 0.9	0.6	1.4
	8	7.4 ± 1.1	7.5 ± 1.3	0.1	1.6
t-PA activity (IU/mL)	**0**	0.5 ± 0.1	0.6 ± 0.1	0.0	0.2
	2	1.3 ± 0.5	1.1 ± 0.4	0.3	0.6
	4	2.0 ± 0.6	1.7 ± 0.9	0.4	1.2
	8	3.4 ± 1.1	3.9 ± 1.4	0.3	1.4
Absolute forearm blood flow (mL/100 mL/min)	**0**	3.5 ± 0.3	3.2 ± 0.3	0.3	0.6
	2	11.2 ± 0.7	10.3 ± 1.3	0.9	3.0
	4	14.4 ± 1.8	12.6 ± 1.5	1.7	4.6
	8	15.7 ± 1.1	14.9 ± 1.5	0.8	3.4
Percentage change in forearm blood flow	**0**	-	-	-	-
	2	206 ± 20	240 ± 30	35	48
	4	300 ± 39	300 ± 36	0	68
	8	402 ± 49	384 ± 58	17	76

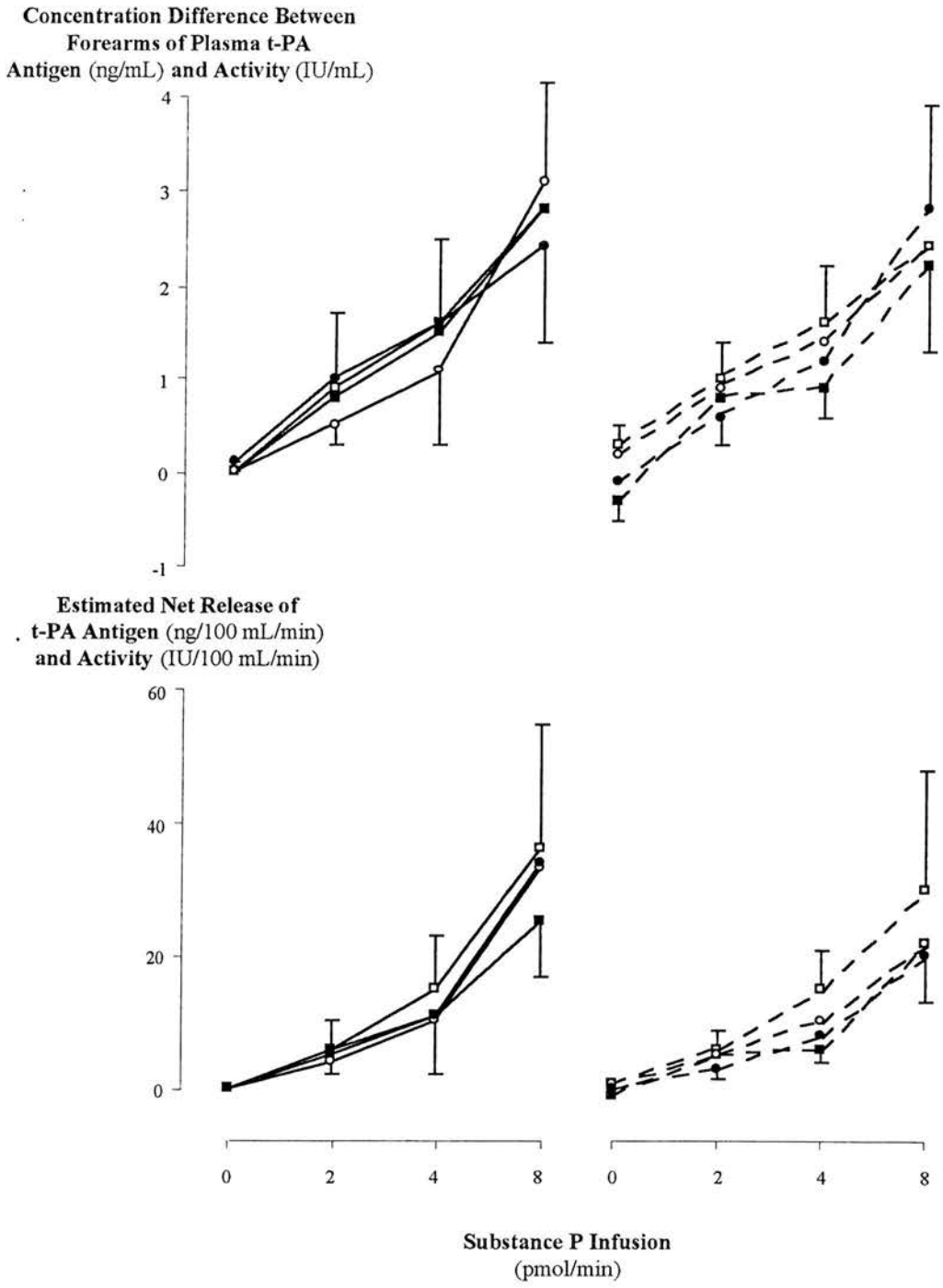


Figure 9.2
Concentration difference between infused and non-infused forearms (upper panels) and estimated net release (lower panels) of plasma t-PA antigen (solid lines; left panels) and activity (dashed lines; right panels) during substance P administration in controls (open circles), and patients at baseline (closed circles), and during placebo (open squares) and pravastatin (closed squares) therapy.
$p<0.001$ for each response (one-way ANOVA)

9.5 DISCUSSION

Despite impaired endothelium-dependent forearm vasodilatation, we have shown that, in patients with hypercholesterolaemia, intra-brachial substance P infusions are associated with a normal capacity to release t-PA acutely. Moreover, pravastatin therapy, sufficient to reduce cholesterol concentrations by 22%, had no significant effects on acute t-PA release. This suggests that, despite the presence of endothelial dysfunction, hypercholesterolaemia does not influence the acute fibrinolytic capacity of the endothelium, and that the preventative benefits of lipid lowering therapy are unlikely to be mediated by improvements in endogenous fibrinolysis.

Consistent with previous findings [Casino *et al* 1995], we have demonstrated that, in patients with hypercholesterolaemia, there is an impairment of endothelium-dependent vasodilatation in response to substance P infusion. However, in contrast to the marked impairment of t-PA release that we have recently described in cigarette smokers [Newby *et al* 1999a], hypercholesterolaemia and lipid lowering therapy do not appear to influence substance P induced t-PA release. Taken together, our findings suggest that although smoking is associated with impaired endogenous fibrinolysis, hypercholesterolaemia is not. This is consistent with the observations that the patency rate of the infarct related artery following thrombolytic therapy during myocardial infarction is enhanced in cigarette smokers [Mueller *et al* 1992; Barbash *et al* 1993; Zahger *et al* 1995] but not in patients with hypercholesterolaemia [De Chillou *et al* 1996], and that cigarette smoking is associated with thrombotic occlusion whereas hypercholesterolaemia is linked to atherogenesis and plaque

rupture [Burke *et al* 1997]. This would also indicate that endothelial dysfunction can be manifest in separate distinct pathways depending upon the nature of the insult.

The WOSCOPS study [Shepherd *et al* 1995] was the first major randomised controlled trial to show the primary preventative benefits of lipid lowering therapy. We, therefore, chose to examine the effects of pravastatin 40 mg daily on endothelial and fibrinolytic function in a well defined and otherwise healthy hypercholesterolaemic population. Although the total cholesterol was reduced by 22% and pravastatin was used at doses which have been shown to confer major preventative benefits in several large scale clinical trials [Shepherd *et al* 1995; Sachs *et al* 1996; The LIPID Study Group 1998], the total serum cholesterol concentration remained significantly higher than the normocholesterolemic population. It may be that a greater reduction in cholesterol concentrations may have facilitated a significant improvement in endothelium-dependent vasodilatation. However, the mean cholesterol concentrations of the patients in the WOSCOPS study were similar to the present study and the relative risk reduction in ischaemic events is the same across a broad range of cholesterol concentrations [The LIPID Study Group 1998]. Moreover, it would appear that statins do not just lower cholesterol and may have many ancillary vascular actions [Vaughan *et al* 1996]. Finally, since we did not observe a significant difference in t-PA release between hypercholesterolaemic and normocholesterolaemic subjects, it is unlikely that additional reductions in lipid concentrations, using higher doses of pravastatin or more potent statins, would influence the acute release of t-PA.

The influence of lipid lowering therapy on endothelial dysfunction was initially studied in patients with hypercholesterolaemia following 3 to 6 months of treatment [Egashira *et al* 1994; Stroes *et al* 1995]. However, more recent studies have demonstrated that endothelial dysfunction can be reversed by six [Koh *et al* 1999] or even four weeks [O'Driscoll *et* al 1997] of statin therapy. Indeed, two hours treatment with plasma LDL apheresis is associated with rapid and immediate reversal of endothelial dysfunction [Tamai *et al* 1997]. It is, therefore, unlikely that the absence of an effect of pravastatin therapy is due to the length of treatment. Moreover, in contrast to previous studies, we have used a randomised double blind crossover study design which provides a greater sensitivity to detect potential differences in responses. The coefficients of reproducibility are consistent with our previous studies [Newby *et al* 1997a; Newby *et al* 1997b] and indicate a power sufficient to detect an ~20% change in blood flow and fibrinolytic responses.

In summary, whereas endothelium-dependent vasodilatation is abnormal in both cigarette smoking and hypercholesterolaemia, acute t-PA release appears to be impaired only by cigarette smoking [Newby *et al* 1999a]. These observations support the concept that endothelial dysfunction is not a homogenous condition and may be manifested in differing ways depending on the nature of the injury. Moreover, the benefits of lipid lowering therapy in the primary [Shepherd *et al* 1995] and secondary [Sachs *et al* 1996; The LIPID Study Group 1998] preventation of coronary events are unlikely to be mediated by improvements in endogenous fibrinolysis.

CHAPTER 10

CORONARY ATHEROSCLEROSIS AND CIGARETTE SMOKING IMPAIR CORONARY TISSUE PLASMINOGEN ACTIVATOR RELEASE: DIRECT ASSOCIATION BETWEEN ENDOTHELIAL DYSFUNCTION AND ATHEROTHROMBOSIS

Newby DE, McLeod AL, Uren NG, Flint L, Ludlam CA,
Webb DJ, Fox KAA, Boon NA. Impaired coronary fibrinolytic capacity is associated with coronary atherosclerosis and cigarette smoking:
direct association between endothelial dysfunction and atherothrombosis.
Under submission.

10.1 SUMMARY

The objective of the study was to establish the relationship between the volume of proximal coronary artery atheroma, the presence of associated risk factors and the stimulated release of tissue plasminogen activator (t-PA) from the heart. Following diagnostic coronary angiography in 25 patients, the left anterior descending artery (LAD) was instrumented and the proximal LAD plaque volume was determined using intravascular ultrasound (IVUS). Blood flow and fibrinolytic responses to selective LAD infusion of saline, substance P (10-40 pmol/min) and sodium nitroprusside (5-20 μg/min) were measured using intracoronary IVUS and Doppler, combined with arterial and coronary sinus blood sampling. Mean plaque burden was 5.5 ± 0.8 mm^3 per mm of vessel (range 0.6-13.7). LAD blood flow increased with both substance P and sodium nitroprusside ($p<0.001$), although coronary sinus plasma t-PA antigen and activity concentrations increased only during substance P infusion ($p\leq0.006$ for both). There was a significant inverse correlation between the LAD plaque burden and release of active t-PA ($r=-0.61$, $p=0.003$). Cigarette smoking, but no other risk factor, was associated with an impairment of coronary release of active t-PA (smokers, 42 ± 21 *versus* nonsmokers, 202 ± 73 $IU.min^{-1}$; $p=0.01$). We have found an association between both the coronary atheromatous plaque burden and smoking habit with the acute local fibrinolytic capacity of the heart. These important findings potentially provide a direct link between endogenous fibrinolysis, endothelial dysfunction and atherothrombosis.

10.2 INTRODUCTION

The endothelium plays a vital role in the control of blood flow, coagulation, fibrinolysis and inflammation. Consequently, the maintenance and regulation of tissue perfusion critically depends upon the integrity of endothelial function and the release of potent endothelium-derived factors. Following the seminal work of Furchgott and Zawadski [Furchgott & Zawadski 1980], it has been widely recognised that an array of mediators can influence vascular tone through endothelium-dependent actions. There is an extensive body of evidence to show that endothelium-dependent vasomotion is abnormal in patients with atherosclerosis [Ludmer *et al* 1986] and its associated risk factors [Celermajer *et al* 1992; Chowieńczyk *et al* 1992; Celermajer *et al* 1996]. However, whilst endothelium-dependent vasomotion is an important indicator of endothelial cell function, it is an indirect surrogate measure of the central pathophysiological role of the endothelium in atherothrombosis.

Acute rupture or erosion of a coronary atheromatous plaque and subsequent coronary artery thrombosis causes the majority of sudden cardiac deaths and myocardial infarctions [Burke *et al* 1997; Davies 2000]. Small areas of denudation and thrombus deposition are a common finding on the surface of atheromatous plaques and are usually sub-clinical [Davies 2000]. However, in the presence of an imbalance in the fibrinolytic system, such microthrombi may propagate, ultimately leading to arterial occlusion [Rosenberg & Aird 1999]. Indeed, genetic murine models indicate that t-

PA deficiency is associated with myocardial necrosis and the development of regional wall motion abnormalities [Christie *et al* 1999].

In the Northwick Park Heart Study [Meade *et al* 1993], low basal plasma fibrinolytic activity was a leading determinant of the risk of sustaining a myocardial infarction or sudden cardiac death in younger men. Moreover, a reduction in exercise induced release of t-PA is associated with an increased incidence of major adverse cardiac events in patients with stable angina pectoris [Held *et al* 1997]. Recently, Rosenberg and Aird [Rosenberg & Aird 1999] have postulated that vascular-bed-specific defects in haemostasis exist, and that coronary thrombosis critically depends on the local fibrinolytic balance. However, there have been no clinical studies to date which have directly assessed the acute local fibrinolytic capacity of the coronary vascular bed in patients with coronary artery disease.

Using forearm venous occlusion plethysmography and the endothelium-dependent agonist, substance P, we have recently developed and characterised a novel model of assessing the acute release of endogenous t-PA *in vivo* in man [Newby *et al* 1997b]. This has enabled us to demonstrate that cigarette smoking is associated with an impairment of acute t-PA release [Newby *et al* 1999a] although this has yet to be confirmed in the coronary circulation. The aims of the present study were: first, to apply this approach to the coronary circulation and thereby establish a method of assessing acute coronary t-PA release; second, to determine the relationship between the extent of coronary artery atheroma, quantified by intravascular ultrasound

(IVUS), and the acute fibrinolytic capacity of the coronary vascular bed; and third, to determine if cigarette smoking impairs coronary as well as forearm t-PA release.

10.3 METHODS

10.3.1 Patient Selection

Patients undergoing elective coronary angiography were recruited unless they had significant aortic stenosis, severe left ventricular dysfunction or recent (<3 months) myocardial infarction. Following angiography, patients were excluded if they had significant left main stem disease or a minimal luminal diameter of less than 2 mm in the proximal left anterior descending coronary artery. All patients had their risk factor profile determined by standard clinical criteria: smoking habit (current/ex-smoker or non-smoker), family history of premature (male: <50 years, female: <55 years) coronary artery disease, and a history of hypertension (systolic blood pressure > 160 mmHg or diastolic blood pressure >90 mmHg on 3 or more occasions), diabetes mellitus (fasting blood glucose concentration >7.8 mmol/L or >11.1 mmol/L 2 hours after 75 g glucose load) or hypercholesterolaemia (total serum cholesterol concentration >5.0 mmol/L). Since many patients discontinued a regular smoking habit just prior to angiography, current and ex-smokers were considered as a single group. The study 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.

10.3.2 Study Protocol

All patients discontinued their medication on the study day, attended fasted and underwent diagnostic coronary angiography at 08.00 h. Following angiography, patients fulfilling the entry criteria were entered into the study and received 5,000 IU

of intravenous heparin. Cannulation of the coronary sinus was established from the femoral vein using a preformed specific 6 F catheter (modified Simmons Torcon NB catheter, HNB6.0-NT-100-PW-2S-112393-BH) [Katritsis & Webb-Peploe 1997]. To avoid atrial blood mixing, the catheter was advanced, sometimes with the assistance of a guide wire, deep into the ostium and beyond the posterior interventricular vein. Stable and selective cannulation of the coronary sinus was achieved in all but three subjects. Arterial samples were obtained through an 8 F haemostatic sheath placed in the right femoral artery.

The left coronary artery was cannulated with a 7 F guiding catheter and a 0.014 inch 12.5 MHz Doppler wire (Flowire™, Cardiometrics, Endosonics, Rancho Cordova, CA, USA) was passed into the left anterior descending coronary artery. A 3.2 F Ultracross™ 20 MHz IVUS imaging catheter (SCIMED®, Boston Scientific Corporation, MN, USA) was advanced into the left anterior descending coronary artery over the guide wire. The IVUS examination of the proximal artery was performed at 0.5 mm/s using a motorised pullback device (Boston Scientific Corp) whilst recording the ultrasound images onto S-VHS tape using the Clearview™ ultrasonogram (Boston Scientific Corp). Following the pullback examination, the IVUS imaging catheter was repositioned just distal to the ostium of the left anterior descending artery. The Doppler guide wire was retracted to the tip of the imaging catheter and maintained in a stable position by the short monorail segment of the IVUS catheter [Schwarzacher *et al* 2000]. The average peak velocity of the Doppler signal was recorded onto S-VHS tape using the Flomap™ (Cardiometrics) Doppler velocimeter.

10.3.3 Drug Administration

Pharmaceutical-grade substance P (Clinalfa AG, Läufelfingen, Switzerland), an endothelium and neurokinin type 1 receptor dependent vasodilator [Newby *et al* 1999c], and sodium nitroprusside (David Bull Laboratories, Warwick, U.K.), an endothelium independent vasodilator, were administered following dissolution in saline (0.9%: Baxter Healthcare Ltd, Thetford, UK). Five minute infusions were administered using an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK) at 1 mL/min via the IVUS catheter flush port [Schwarzacher *et al* 2000]. The agents were given in the following order: saline, substance P 10 pmol/mL, substance P 20 pmol/mL, substance P 40 pmol/mL, sodium nitroprusside 5 μg/mL and sodium nitroprusside 20 μg/mL. Due to limitations in procedural time, the sodium nitroprusside responses were determined in 14 patients only.

10.3.4 Measurement Of Plaque Volume And Coronary Blood Flow

Computerised three dimensional reconstructions of the proximal left anterior descending coronary artery were performed off-line by a single blinded operator using the TomTec computer system (Echoscan, TomTec Imaging Systems, Unterschleissheim, Germany). The proximal atheromatous plaque volume was calculated using a well validated edge detection algorithm [von Birgelen *et al* 1996a; von Birgelen *et al* 1997]. The planar contours were checked by the operator and, if necessary, edited. The plaque area was defined as the region between the luminal border and the echogenic interface of the external elastic lamina as previously described [Di Mario *et al* 1998; von Birgelen *et al* 1996b].

The left anterior descending coronary artery cross-sectional area was measured using computerised planimetry (Clearview™, Boston Scientific Inc) of the vessel lumen. The IVUS images were gated to the electrocardiogram and measurements were made at the onset of the QRS complex. Blood flow velocity was determined using average peak velocity of the Doppler signal (Flopmap™, Cardiometrics). Blood flow in the left anterior descending coronary artery was determined from the mean of five measurements made in the final minute of each infusion period and was defined as [Doucette *et al* 1992]:

$$\text{Coronary blood flow (mL/min)} = \text{CSA} \cdot \frac{\text{APV}}{2} \cdot \frac{60}{100}$$

where CSA = cross-sectional area (mm^2) and APV = average peak velocity (cm/s).

10.3.5 Blood Sampling And Plasma Assays

Ten mL of arterial and coronary sinus blood were obtained simultaneously at the end of each infusion period, collected into acidified buffered citrate (Biopool® Stabilyte™, Umeå, Sweden) and citrate (Monovette®, Sarstedt, Nümbrecht, Germany) tubes, and kept on ice before being centrifuged at 2,000 *g* for 30 minutes at 4°C. Platelet free plasma was decanted and stored at -80°C before assay. Coronary sinus oxygen saturations were determined at the end of each infusion period using an automated oximeter (Oxicam™ 300, Watco Services, Basingstoke, UK).

10.3.6 Data Analysis And Statistics

Coronary t-PA release was defined as the product of the left anterior descending artery plasma flow (based on the haematocrit, Hct and the left anterior descending coronary blood flow, CBF) and the plasma arterial ($[t\text{-}PA]_{Art}$) and coronary sinus ($[t\text{-}PA]_{Ven}$) concentration differences.

$$\text{Estimated net coronary t-PA release} = CBF \times \{1\text{-}Hct\} \times \{[t\text{-}PA]_{Ven} - [t\text{-}PA]_{Art}\}$$

In order to compare vasomotor and fibrinolytic responses with proximal atheromatous plaque volume, the area under the curve (AUC) was calculated for each response: coronary blood flow, plasma arterial and coronary sinus t-PA concentration differences, and estimated net t-PA release.

Data were examined by analysis of variance (ANOVA) with repeated measures, two tailed paired and unpaired Student's *t*-test, and univariate and multivariate regression analysis using StatView *v*5.0.1 (SAS Institute Inc., Cary, North Carolina, USA). All results are expressed as mean ± standard error of the mean. Statistical significance was taken at the 5% level.

10.4 RESULTS

Baseline patient characteristics are shown in Table 1. In keeping with the anticipated profile of patients undergoing coronary angiography, the study population was predominantly middle-aged, male and had a combination of risk factors. Throughout the study there were no significant changes in heart rate, mean arterial pressure or haematocrit (0.40 ± 0.01).

10.4.1 Plaque Volume And Blood Flow Responses

The proximal 29 ± 1 mm of the left anterior descending coronary artery were reconstructed and found to contain 160 ± 24 mm^3 of atheromatous plaque: a plaque burden of 5.5 ± 0.8 mm^3 per mm of vessel (range, 0.6 to 13.7). There was a significant linear correlation between the plaque burden and the serum total cholesterol: HDL-cholesterol ratio ($r=0.55$, $p=0.004$).

Left anterior descending coronary artery blood flow increased with both substance P and sodium nitroprusside infusion ($p<0.001$, ANOVA; see Table 2). There was a significant linear correlation between the percentage increase in coronary sinus oxygen saturations and left anterior descending coronary artery flow ($r=0.46$, $p<0.001$). However, there was no correlation between the plaque burden and the AUC for the coronary blood flow responses to substance P or sodium nitroprusside infusion. In contrast, there was an association between the number of risk factors for atherosclerosis and the coronary blood flow responses to substance P (Figure 1: $r=-0.42$, $p<0.05$).

Table 10.1
Patient characteristics

Number		25	
Age		56 ± 2	years
Sex		17	male
Body Mass Index		28 ± 1	kg/m^2
Risk Factors	**Current/Ex-smoker**	17	
	Hypertension	6	
	Diabetes Mellitus	3	
	Hyperlipidaemia	19	
	Family History	13	
Serum Lipid Profile	**Total Cholesterol**	5.8 ± 0.3	(mmol/L)
	LDL Cholesterol	3.2 ± 0.2	(mmol/L)
	HDL Cholesterol	1.3 ± 0.1	(mmol/L)
	Total/HDL Cholesterol Ratio	4.9 ± 0.5	
	Triglycerides	2.3 ± 0.1	(mmol/L)
Medical therapy	**Aspirin**	22	
	ß-Adrenergic Blockade	18	
	Calcium Antagonism	12	
	Long-acting Nitrate	8	
	Lipid Lowering Therapy	16	
	ACE Inhibition	3	
	Diuretics	3	
Previous Myocardial Infarction		6	
Non-Invasive Testing	**Low Risk**	9	
	High Risk	12	
	Not Performed	4	
Angiographic Data	**Good Left Ventricular Function**	24	
	Normal/Mild Disease	8	
	Single Vessel Disease	9	
	Two Vessel Disease	6	
	Three Vessel Disease	2	

Table 10.2
Haemodynamics, coronary blood flow, tissue plasminogen activator (t-PA) release and plasma plasminogen activator inhibitor type 1 (PAI-1) concentrations during substance P and sodium nitroprusside infusion

		Saline	Substance P (pmol/min)			Saline	Sodium Nitroprusside (μg/min)	
			10	20	40		5	20
Heart Rate	(/min)	65 ± 2	64 ± 3	64 ± 3	64 ± 3	69 ± 3	69 ± 3	68 ± 4
Mean Arterial Pressure	(mmHg)	98 ± 3	99 ± 3	98 ± 3	98 ± 3	94 ± 3	96 ± 5	90 ± 4
Coronary Sinus Oxygen Saturation	(%)	44 ± 2	48 ± 2	48 ± 3	46 ± 2	44 ± 3	-	54 ±4
Intravascular Ultrasound and Doppler								
Luminal Cross-sectional Area	(mm^2)	15.7 ± 1.0	16.4 ± 1.0‡	16.5 ± 1.0‡	16.9 ± 1.0‡	14.9 ± 1.1	15.4 ± 1.1‡	16.1 ± 1.3†
Average Peak Velocity	(cm/s)	21.4 ± 1.6	27.3 ± 2.2‡	27.5 ± 2.4‡	28.0 ± 2.3‡	22.7 ± 2.2	27.0 ± 2.2†	47.1 ± 4.6‡
Absolute Coronary Blood Flow	(mL/min)	103 ± 12	138 ± 18‡	140 ± 19‡	143 ± 18‡	109 ± 18	131 ± 20†	242 ± 42†
Change in Coronary Blood Flow	(%)	0	36 ± 6‡	37 ± 7‡	46 ± 10‡	0	29 ± 9†	140 ± 24‡
Plasma t-PA Antigen								
Arteriovenous Concentration Difference	(ng/mL)	0.3 ± 0.2	0.8 ± 0.3	0.7 ± 0.3	1.0 ± 0.3*	0.0 ± 0.2	-	-0.1 ± 0.4
Estimated Net Release	(ng/min)	12 ± 11	22 ± 31	19 ± 36	74 ± 24†	1 ± 15	-	23 ± 82
Plasma t-PA Activity								
Arteriovenous Concentration Difference	(IU/mL)	0.1 ± 0.1	0.6 ± 0.2†	0.2 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	-	-0.0 ± 0.0
Estimated Net Release	(IU/min)	6 ± 3	45 ± 15†	15 ± 8	27 ± 14	12 ± 7	-	-10 ± 9
Plasma PAI-1 Antigen								
Coronary Sinus Concentration	(ng/mL)	69 ± 8	68 ± 7	67 ± 7	66 ± 7	68 ± 11	-	72 ± 12
Arterial Concentration	(ng/mL)	69 ± 8	69 ± 8	66 ± 7	66 ± 7	70 ± 12	-	72 ± 12
Plasma PAI-1 Activity								
Coronary Sinus Concentration	(AU/mL)	19 ± 4	19 ± 4	17 ± 4	16 ± 4	18 ± 8	-	17 ± 8
Arterial Concentration	(AU/mL)	18 ± 3	18 ± 3	18 ± 4	18 ± 3	15 ± 6	-	18 ± 8

n=14 for sodium nitroprusside responses
n=22 for fibrinolytic parameters (except PAI-1 activity, n=13)
*p≤0.05; †p≤0.01; ‡p≤0.001 (*versus* baseline, paired *t*-test)

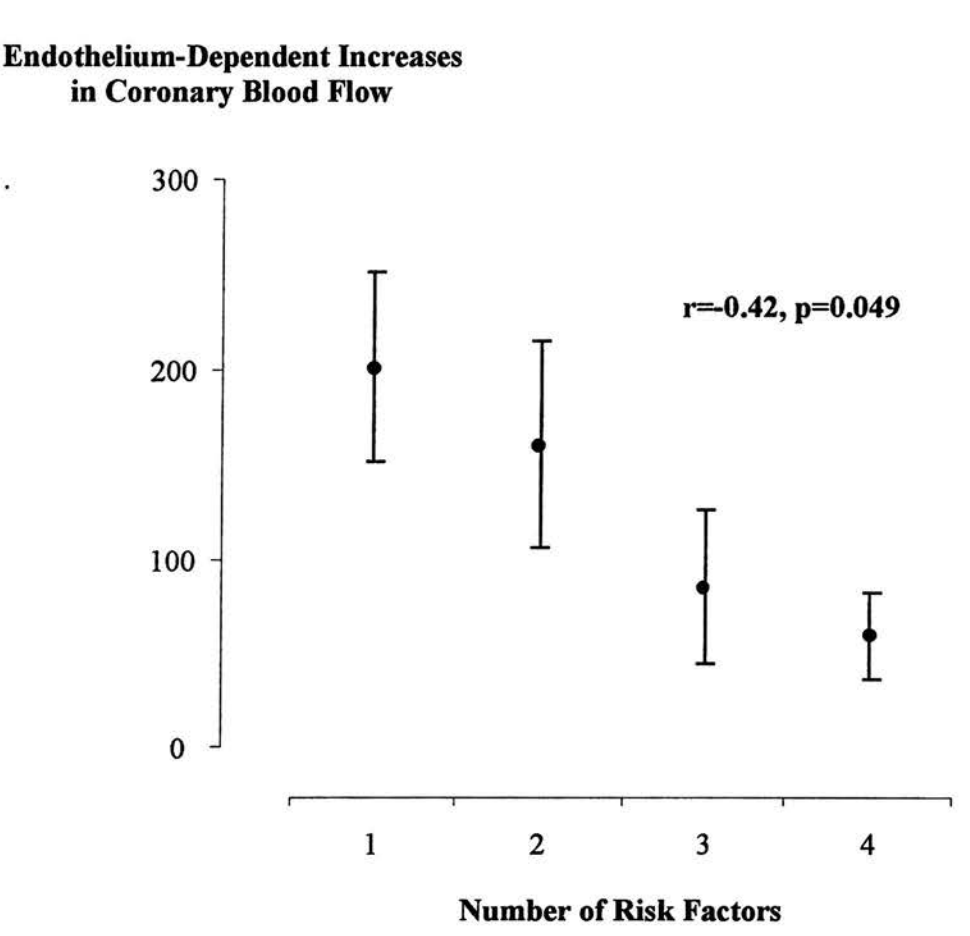


Figure 10.1
Prevalence of risk factors and influence on coronary blood flow responses to substance P infusion.

10.4.2 Plasma Fibrinolytic Parameters

There was a significant rise in plasma t-PA antigen and activity concentrations from the coronary sinus during substance P infusion (Figure 2: ANOVA, $p<0.001$ and $p<0.006$ respectively) but not during sodium nitroprusside infusion. There was a significant inverse correlation between the plaque burden and the AUC for active t-PA release (Figure 3: $r=-0.61$, $p=0.003$), and a trend for the AUC for t-PA antigen release ($r=-0.34$, $p=0.15$). There was also an inverse linear correlation between the basal coronary sinus plasma t-PA antigen concentration and the AUC for active t-PA release ($r=-0.58$, $p<0.005$).

Ex- and current cigarette smokers had a higher basal plasma t-PA antigen concentration and an impaired active t-PA response to substance P infusion in comparison to non-smokers despite similar plasma PAI-1 concentrations and coronary arterial plaque burden (Table 3). Subgroup analysis demonstrated that current smokers ($n=4$) had similar release of active t-PA compared to ex-smokers ($n=13$): 49 ± 32 and 40 ± 28 IU/min respectively. Hypercholesterolaemia, hypertension, diabetes mellitus and a family history of premature coronary artery disease had no influence on t-PA release.

There were no significant changes in plasma PAI-1 antigen and activity concentrations throughout the study (Table 2). Basal coronary sinus plasma PAI-1 antigen concentrations correlated positively with plaque burden ($r=0.47$, $p<0.03$) and negatively with release of active t-PA ($r=-0.44$, $p=0.04$).

Multivariate regression analysis identified plaque burden and basal coronary sinus t-PA antigen concentrations as the independent variables that were significantly associated with release of active t-PA ($p \leq 0.02$ for both).

Table 10.3
Influence of smoking status on the tissue plasminogen activator (t-PA) release, plasminogen activator inhibitor type 1 (PAI-1) and endothelium-dependent increases in coronary blood flow.

	Smokers	Non-Smokers	
Plaque Burden	5.8 ± 0.8	4.5 ± 1.6	(mm^3/mm)
Coronary Blood Flow			
Basal Blood Flow	104 ± 16	93 ± 15	(mL/min)
Percentage Increase*	129 ± 28	125 ± 42	(%)
Plasma t-PA Antigen			
Basal Coronary Sinus Concentration	8.7 ± 0.6	6.3 ± 0.7†	(ng/mL)
Basal Arterial Concentration	8.3 ± 0.6	6.3 ± 0.8‡	(ng/mL)
Arteriovenous Concentration Difference*	2.8 ± 1.0	2.6 ± 1.2	(ng/mL)
Estimated Net Release*	98 ± 108	190 ± 85	(ng/min)
Plasma t-PA Activity			
Basal Coronary Sinus Concentration	0.5 ± 0.1	0.6 ± 0.2	(IU/mL)
Basal Arterial Concentration	0.4 ± 0.1	0.5 ± 0.2	(IU/mL)
Arteriovenous Concentration Difference*	0.6 ± 0.3	2.8 ± 1.1†	(IU/mL)
Estimated Net Release*	42 ± 21	202 ± 73†	(IU/min)
Plasma PAI-1 Antigen			
Basal Coronary Sinus Concentration	73 ± 9	59 ± 14	(ng/mL)
Basal Arterial Concentration	73 ± 9	60 ± 15	(ng/mL)
Plasma PAI-1 Activity			
Basal Coronary Sinus Concentration	17 ± 5	24 ± 8	(AU/mL)
Basal Arterial Concentration	17 ± 4	23 ± 8	(AU/mL)

*Area under the curve
†$p \leq 0.02$; ‡$p = 0.07$ (current/ex-smokers *versus* non-smokers, unpaired *t*-test)

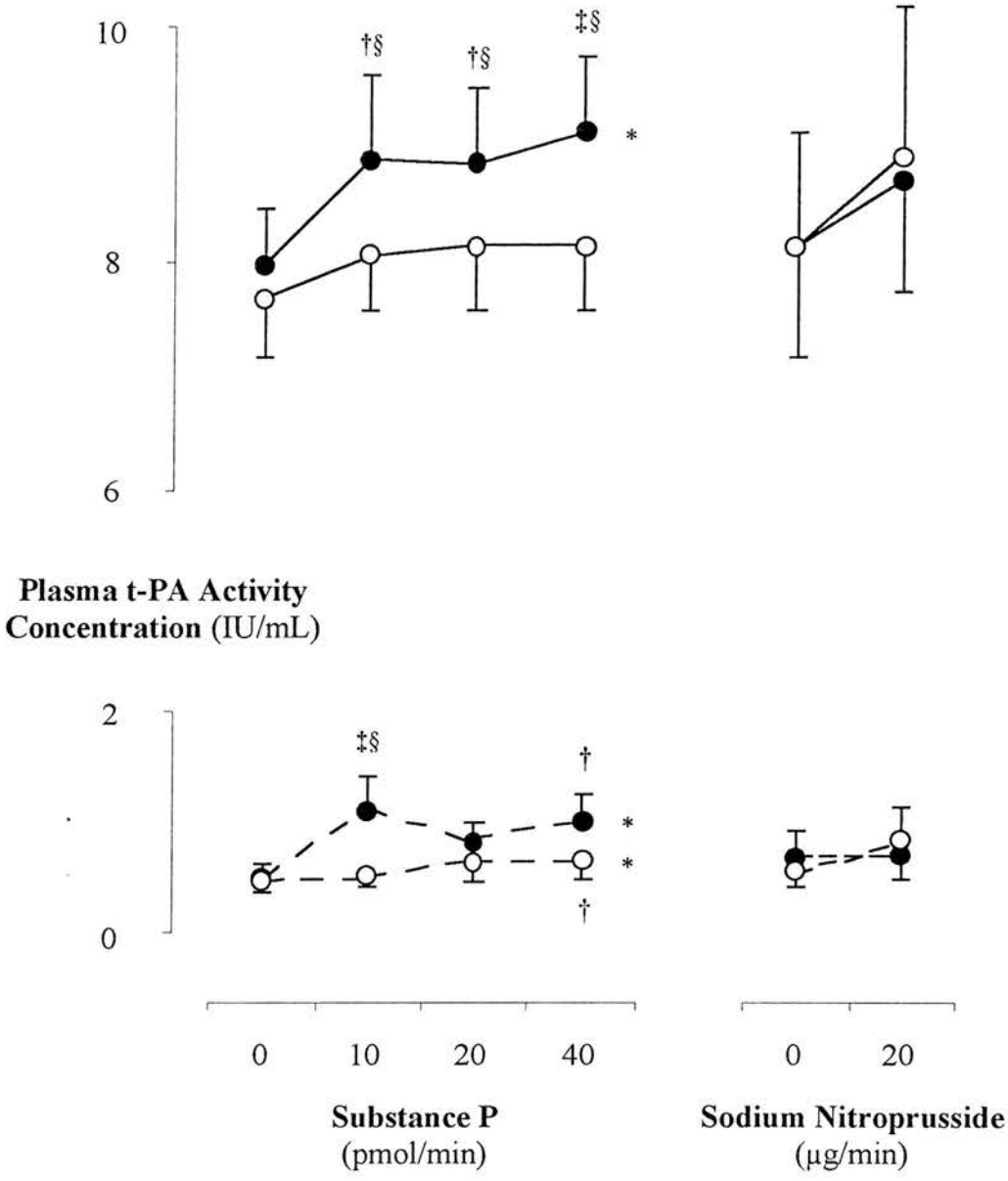


Figure 10.2
Arterial (open circles) and coronary sinus (closed circles) plasma tissue plasminogen activator (t-PA) antigen (solid lines) and activity (dashed lines) concentrations during substance P (left panel) and sodium nitroprusside (right panel) infusion.

*p≤0.007	(ANOVA with repeated measures)
†p≤0.01; ‡p≤0.001	(*versus* baseline, paired *t*-test)
§p≤0.05	(*versus* arterial, paired *t*-test)

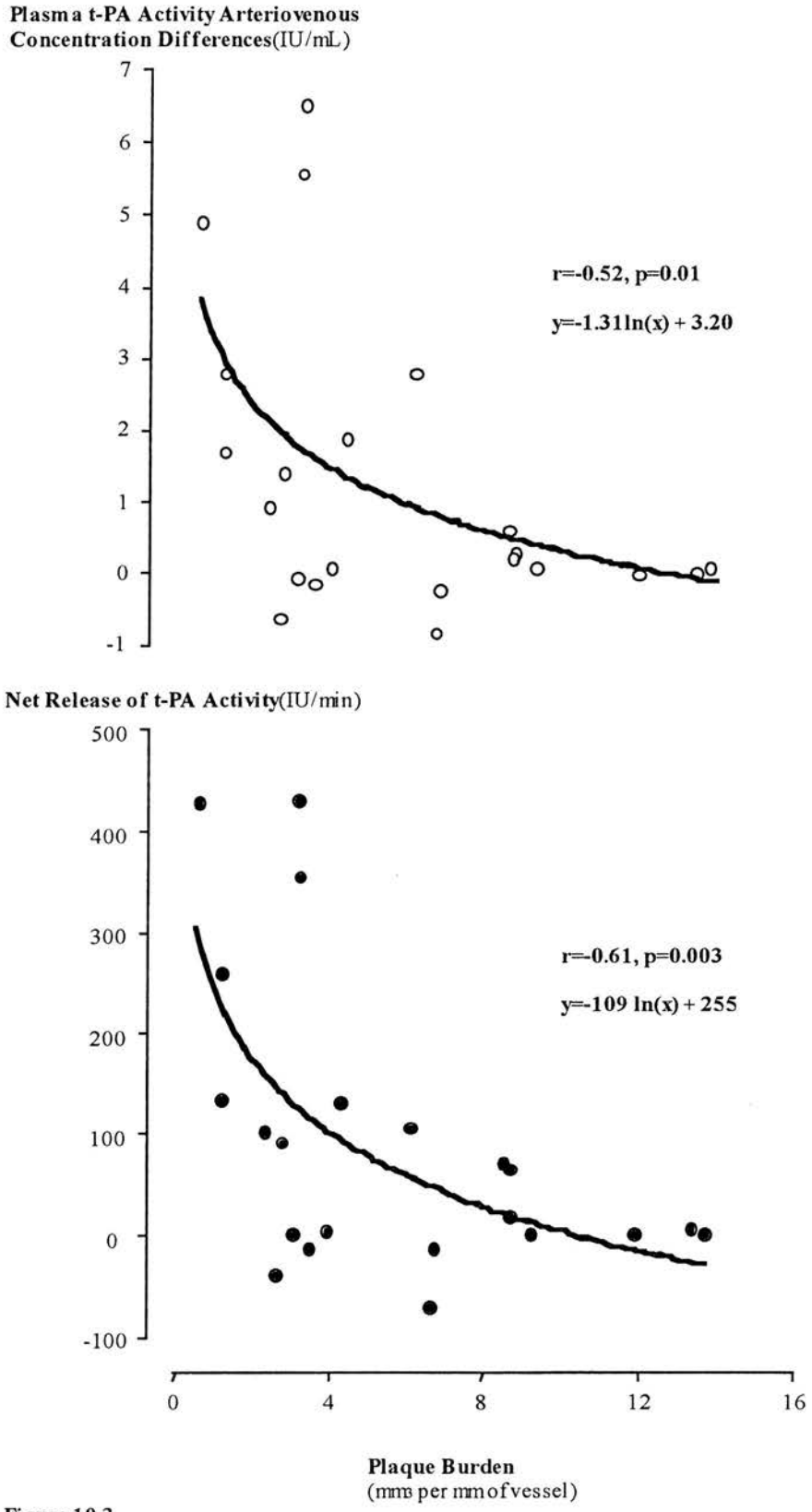


Figure 10.3
Correlation of the plaque burden of the left anterior descending coronary artery with the AUC for the arteriovenous concentration differences (upper panel) and the estimated net release (lower panel) of tissue plasminogen activator (t-PA) activity.

10.5 DISCUSSION

For the first time, we have shown a direct relationship between both the coronary atheromatous plaque burden and smoking habit with the acute fibrinolytic capacity of the heart. These important findings suggest that both atherosclerosis and smoking habit influence the local fibrinolytic balance in the coronary circulation, and provide a direct link between endothelial dysfunction, atherothrombosis and myocardial infarction.

The clinical importance of endogenous t-PA release is exemplified by the high rate of spontaneous reperfusion in the infarct related artery after acute myocardial infarction; occurring in one third of patients within the first 12 hours [De Wood *et al* 1980; Armstrong *et al* 1989; Rentrop *et al* 1989]. The present study is the first to attempt directly to assess the acute release of t-PA in the coronary circulation of patients with stable coronary artery disease. Although thrombin and activated coagulation Factor X may be more physiologically relevant to acute t-PA release than substance P, we have used the latter because its vascular actions are endothelium-dependent [Gross *et al* 1994] and its administration intra-arterially is safe and well tolerated [Crossman *et al* 1989; Newby *et al* 1997a]. Using this approach, we have been able to stimulate the fibrinolytic activity of the coronary vascular bed and demonstrate that this response appears to be sensitive to the presence of atheroma: a rapid decline in release of active t-PA associated with an increasing plaque burden. This reduction in acute fibrinolytic capacity appears to reflect both an impairment of acute t-PA release and an elevation in plasma PAI-1 concentrations. The mechanisms underlying this relationship remain to be

established but are likely to involve chronic endothelial cell injury and an impairment of the L-arginine:nitric oxide pathway [Newby *et al* 1998]. In addition, this association may reflect the chronic stimulation and up-regulation of basal t-PA release secondary to the presence of atheroma [Steins *et al* 1999] and arterial denudation. The subsequent depletion of endothelial cell t-PA stores, the associated increases in PAI-1 concentrations and the overall reduction of the acute dynamic fibrinolytic response, would potentially be detrimental. This hypothesis is consistent with the epidemiological observations of a positive correlation between plasma t-PA and PAI-1 antigen concentrations and future coronary events [Hamsten *et al* 1985; Meade *et al* 1986; Jansson *et al* 1993; Meade *et al* 1993; Ridker *et al* 1993a; Thompson *et al* 1995; Thögersen *et al* 1998] as well as our findings of inverse correlations between the release of active t-PA, and basal coronary sinus t-PA and PAI-1 antigen concentrations.

Questions of cause and effect cannot be resolved by the present study but our observations are also consistent with a reduced fibrinolytic activity causing enhanced atherogenesis. Detailed post-mortem studies have shown that plaque growth is induced by episodic subclinical plaque disruption and thrombus formation [Mann & Davies 1999]. The prolonged presence of residual thrombus over a disrupted or eroded plaque will provoke smooth muscle migration and the production of new connective tissue, leading to plaque expansion [Galis *et al* 1997; Nomura *et al* 1999; Davies 2000]. This is consistent with the enhanced macrovascular fibrin deposition and atherogenesis seen in genetic murine models of t-PA and plasminogen deficiency [Xiao *et al* 1997; Christie *et al* 1999].

In agreement with our previous work in the peripheral circulation [Newby *et al* 1999a], we have also observed an elevated basal plasma t-PA antigen concentration and an impairment in coronary release of active t-PA in cigarette smokers. These observations suggest that the increased risk of coronary thrombosis seen in smokers [Burke *et al* 1997] relates to impaired endogenous fibrinolysis and the propagation of thrombus that would otherwise undergo lysis and remain sub-clinical. Although cigarette smokers have a higher overall mortality from myocardial infarction than non-smokers [Håheim *et al* 1993], the in-hospital mortality is lower [Mueller *et al* 1992; Barbash *et al* 1993; Zahger *et al* 1995]. This so-called "smokers' paradox" can be explained by the observation that the infarct related artery is more than twice as likely to become patent in current smokers compared to non-smokers following thrombolytic therapy for acute myocardial infarction [Gomez *et al* 1993; Zahger *et al* 1995; De Chillou *et al* 1996]. Indeed, it has been suggested that thrombolytic therapy should only be given to smokers, and that alternative strategies such as primary angioplasty used in non-smokers [Bowers *et al* 1996]. Our findings may account for these observations since it might be anticipated that patients with impaired endothelial cell t-PA release would benefit most from thrombolytic therapy whilst those with a normal endogenous fibrinolytic capacity are more likely to have coronary thrombus resistant to fibrinolysis.

Previous studies [Zeiher *et al* 1991; Kuga *et al* 1995] have suggested that there is a direct association between coronary atherosclerosis and endothelium-dependent vasodilatation. These studies have assessed the vasomotor responses and the extent

of atherosclerosis using quantitative coronary angiography (QCA). There are several inherent limitations and inaccuracies of QCA [Newby 2000b] which permits only crude estimates of plaque load [De Feyter *et al* 1991; Topol & Nissen 1995] and underestimates the coronary artery luminal diameter and cross-sectional area [De Scheerder *et al* 1994; Escaned *et al* 1996; Moussa *et al* 1999]. These inaccuracies occur because QCA can only assess the arterial lumen and the arterial wall is extrapolated from a reference segment and does not take account of "Glagovian" remodelling [Glagov *et al* 1987; Escaned *et al* 1996]. In contrast, IVUS provides a more accurate assessment of intracoronary plaque volume that has been extensively validated [Hausmann *et al* 1994; von Birgelen *et al* 1997]. Using this methodology, we have failed to detect a direct association between the atherosclerotic plaque burden and substance P induced vasodilatation. This is, in part, likely to reflect the independent influence of atherosclerotic risk factors on endothelium-dependent vasomotion and is borne out by the correlation of the substance P vasodilatation and the prevalence of these risk factors.

10.5.1 Study Limitations

The proportionate increase in coronary plasma t-PA concentrations appears to be more modest than that obtained from the forearm circulation in healthy volunteers [Newby *et al* 1997b] and is likely to reflect a number of factors including the direct influence of atherosclerosis. Because the coronary sinus also drains regions of the heart outwith the left anterior descending coronary artery territory, the current methodology will also tend to underestimate the net coronary release of t-PA. Moreover, regional and visceral differences in t-PA release do exist such that the

upper limbs release four times the amount of the lower limbs [Keber 1988], and unlike the systemic circulation, the liver and kidney do not acutely release t-PA when challenged with desmopressin [Brommer *et al* 1988].

Since the estimated net t-PA release was defined as a product of the arteriovenous difference and plasma flow, impaired endothelial vasodilatation may contribute to the observed reduction in net release of active t-PA. However, this is unlikely for two reasons. First, there was an independent correlation between the arteriovenous difference and the atheromatous plaque burden. Theoretically, smaller increases in blood flow would be associated with greater increases in the arteriovenous concentration differences and obscure this latter association. Second, we failed to detect a significant correlation between the atheromatous plaque burden and substance P induced vasodilatation.

A clear incremental dose response to substance P infusion was not observed. The doses of substance P were chosen on the basis of the effective end-organ concentrations associated with t-PA release in the forearm [Newby *et al* 1999a]. Given that the left anterior descending coronary artery blood flow is approximately 4-5-fold forearm blood flow, we utilised substance P at doses of 10-40 pmol/min (c.f. 2-8 pmol/min in the forearm) which are at the limit of breakthrough systemic effects [Crossman *et al* 1989]. These intracoronary doses have previously been shown to have a similar plateau of vasodilatation responses [Crossman *et al* 1989] and are consistent with our own findings.

In conclusion, we have demonstrated, for the first time, a direct association between both the coronary atheromatous plaque burden and smoking habit with the acute local fibrinolytic capacity of the coronary circulation. These important findings may provide a direct link between endothelial dysfunction and atherothrombosis. Interventions targeted at the enhancement of the local fibrinolytic capacity will potentially be of major importance.

CHAPTER 11

CONCLUSIONS AND FUTURE DIRECTIONS

11.1 SUBSTANCE P MEDIATED STIMULATION OF THE ENDOTHELIUM

11.1.1 Substance P Mediated Vasodilatation

In a series of studies, it has been demonstrated that although endogenous substance P does not appear to contribute to the maintenance of peripheral vascular tone or systemic blood pressure, exogenous substance P causes a dose-dependent increase in blood flow through an endothelium- and NK_1 receptor-dependent mechanism. In the human forearm, exogenous substance P administration appears to be well tolerated and causes reproducible blood flow responses both within and between study days.

11.1.2 Substance P Mediated Tissue Plasminogen Activator Release

Substance P mediated vasodilatation is associated with a selective and reproducible forearm release of t-PA at intra-brachial doses of 2 pmol/min or higher. This endothelial cell stimulation is not accompanied by the co-release of other endothelial cell peptidic products, such as PAI-1 or vWf, despite protracted cellular stimulation of up to 2 hours. This indicates that there is a distinct dynamic intracellular storage pool of t-PA. Our findings, therefore, suggest that significant co-localisation of t-PA with vWf in the Weibel Palade bodies [Datta *et al* 1999; Rosnoblet *et al* 1999] is unlikely and is consistent with separate distinct storage granules for these two glycoproteins [Emeis *et al* 1997].

There appears to be a substantial capacity for t-PA release in the forearm circulation. Assuming a forearm volume of 1000 mL, the average release of t-PA with substance

P at 64-128 pmol/min was approximately 10 μg over 10 minutes. Moreover, stimulation of the endothelium over a 2 hour period was associated with a sustained release of t-PA, exceeding 30 μg in some individuals, indicating that there are large intracellular stores which do not become readily depleted. Thus, the endothelium appears to have a vast fibrinolytic reserve which can be rapidly mobilised in the presence of intravascular thrombosis.

11.2 MECHANISMS OF TISSUE PLASMINOGEN ACTIVATOR RELEASE

11.2.1 Selective Tissue Plasminogen Activator Release

The selective endothelial cell release of t-PA is not unique to substance P. Recently, both bradykinin [Brown *et al* 1999; Labinjoh *et al* 2000a] and desmopressin infusions [Wall *et al* 1998; Newby *et al* 2000a] have been shown to induce selective forearm release of t-PA in the absence of a change in PAI-1, vWf or factor VIII:C concentrations. However, it is interesting to note that not all endothelial cell stimulants release t-PA. For example, acetylcholine administration causes no detectable release of t-PA despite marked increases in forearm blood flow [Brown *et al* 1999]. Moreover, endothelin-1 administration is not associated with a significant release of t-PA despite the stimulation of endothelial ET_B receptors [Newby *et al* 1999d]. The reason for these disparities may lie in the mechanisms of action of these compounds and it would appear that different G-protein coupled receptors invoke distinct endothelial cell responses.

Substance P and bradykinin are both inflammatory mediators and, when administered into the forearm, are associated with the formation of cutaneous oedema [Newby *et al* 1997b; Labinjoh *et al* 2000a]. Moreover, desmopressin infusion provokes a systemic inflammatory response which induces a 10-fold increase of the cytokine, interleukin-6 [Newby *et al* 2000a]. It may, therefore, be that provocation of endothelial cell t-PA release requires a specific second messenger signal which involves an inflammatory process.

11.2.2 Cellular Mechanisms Of Tissue Plasminogen Activator Release

Unlike desmopressin [Wall *et al* 1998; Newby *et al* 2000a], substance P induced t-PA release has a rapid onset and offset of action that reaches a maximal effect within a few minutes. The cellular mechanisms involved in the release of t-PA from intracellular storage granules are unclear [Rydholm *et al* 1995] but would appear to involve calcium ions [Tranquille *et al* 1991] and, in part, relate to the action of the L-arginine:nitric oxide system [Newby *et al* 1998]. However, increases in blood flow and nitric oxide release do not *per se* induce t-PA release since infusion of sodium nitroprusside to increase forearm blood flow by more than 600% does not affect t-PA release [Jern *et al* 1994a+b; Newby *et al* 1997b; Stein *et al* 1998].

The endothelium expresses a vast array of G-protein coupled receptors that, when stimulated, can evoke the release of a range of vasoactive mediators, such as prostanoids, nitric oxide and endothelium-derived hyperpolarising factor (EDHF). In contrast, direct local release of t-PA appears to occur only in response to certain agonists, whilst methods of selectively inducing vWf and PAI-1 release have yet to be identified. Thus, the endothelium appears to have a complex and differential response to specific receptor mediated stimuli. It is clear that these intracellular mechanisms and pathways will be central to our understanding and interpretation of the functional studies of the endothelium but they have yet to be precisely characterised.

11.3 ENDOTHELIAL DYSFUNCTION AND IMPAIRED ENDOGENOUS FIBRINOLYSIS

To date, the majority of investigational studies assessing endothelial function have focused upon endothelium-dependent vasomotion and have shown that it is impaired by many of the risk factors associated with coronary artery disease, such as hypercholesterolaemia [Chowienczyk *et al* 1992; Stroes *et al* 1995], diabetes mellitus [Calver *et al* 1992], familial history of atherosclerosis [Celermajer *et al* 1992] and smoking [Celermajer *et al* 1996; Heitzer *et al* 1996a+b; Newby *et al* 1999a]. Endothelial function is also impaired in the coronary circulation of patients with coronary artery disease [Ludmer *et al* 1986] and its risk factors [Vita *et al* 1990] including smoking [Zeiher *et al* 1995]. However, endothelial regulation of vasomotion is but one of the many aspects of endothelial function (Table 1.2) and represents a surrogate marker for the role of the endothelium in atherothrombosis.

It is perhaps remarkable that other aspects of endothelial function, such as haemostasis and fibrinolysis, have been relatively under investigated. Here, the endogenous fibrinolytic capacity of subjects with risk factors for atherosclerosis, namely cigarette smoking and hypercholesterolaemia, and patients undergoing diagnostic coronary angiography with a range of coronary artery disease, have been explored. Endothelial fibrinolytic dysfunction, as manifest by an impairment of endothelial t-PA release, appears to be a feature of cigarette smoking and atherosclerosis but not hypercholesterolaemia. This would indicate that endothelial dysfunction can be manifest in separate distinct pathways depending upon the nature

of the insult. Indeed, given the disparate mechanisms of inducing endothelial cell injury, it would perhaps be surprising if the risk factors for atherosclerosis consistently induced an identical pattern of endothelial dysfunction.

11.3.1 Impaired Endogenous Fibrinolysis And Cigarette Smoking

The components of cigarette smoke that confer this impairment of t-PA release remain to be established. Nicotine has vasoactive effects and may play role, especially given the suggestion that it can induce endothelial dysfunction [Sarabi & Lind 2000]. However, the composition of tobacco smoke is complex and includes a range of potential candidates with vascular and inflammatory actions.

Increased oxidative stress is associated with cigarette smoking and is likely to play a significant role. Antioxidant vitamins have the potential to, at least in part, reverse the endothelial vasomotor dysfunction [Heitzer *et al* 1996a; Neunteufl *et al* 2000] associated with cigarette smoking. The effect of antioxidant intervention, such as vitamins C and E supplementation, on t-PA release in cigarette smokers needs to be addressed. These issues relating to cigarette smoking are the subject of an on-going British Heart Foundation Research Project (PG99110).

11.4 ENDOTHELIAL DYSFUNCTION AND IMPAIRED CORONARY ENDOGENOUS FIBRINOLYSIS

Recently, Rosenberg and Aird [Rosenberg & Aird, 1999] have postulated that vascular-bed-specific defects in haemostasis exist, and that coronary thrombosis critically depends on the local fibrinolytic balance. However, until now, there have been no clinical studies which have directly assessed the acute local fibrinolytic capacity of the coronary vascular bed in patients with coronary artery disease. For the first time, the release of t-PA from the coronary circulation has been characterised using substance P infusion.

The acute fibrinolytic activity of the heart has an inverse correlation with the extent of proximal coronary artery atherosclerosis. The mechanisms underlying this relationship remain to be established but are likely to involve chronic endothelial cell injury and an impairment of the L-arginine:nitric oxide pathway [Newby *et al* 1998]. Alternatively, this association may reflect the chronic stimulation and up-regulation of basal t-PA release secondary to the presence of atheroma [Steins *et al* 1999] and arterial denudation. The subsequent depletion of endothelial cell t-PA stores, and the desensitisation and reduction of the acute fibrinolytic response, would potentially be detrimental. This hypothesis is consistent with the epidemiological observations of a positive correlation between plasma t-PA antigen concentrations and future coronary events [Hamsten *et al* 1985; Meade *et al* 1986; Jansson *et al* 1993; Meade *et al* 1993; Ridker *et al* 1993a; Thompson *et al* 1995; Thögersen *et al* 1998] as well as our

finding of an inverse correlation between basal t-PA antigen concentrations and t-PA activity release.

Questions of cause and effect cannot be resolved by our observations and it remains a possibility that reduced fibrinolytic activity causes enhanced atherogenesis. The prolonged presence of residual thrombus over a disrupted or eroded plaque will provoke smooth muscle migration and the production of new connective tissue, leading to plaque expansion [Galis *et al* 1997; Nomura *et al* 1999; Davies 2000]. This is consistent with the enhanced macrovascular fibrin deposition and atherogenesis seen in genetic murine models of t-PA and plasminogen deficiency [Xiao *et al* 1997; Christie *et al* 1999].

11.4.1 Coronary And Peripheral Endogenous Fibrinolysis

A comparison of the peripheral and coronary vascular fibrinolytic responses to endothelial cell stimulation will elucidate which factors determine both the local and the systemic fibrinolytic capacity. Moreover, such comparisons will ascertain whether the assessment of endothelial function and fibrinolytic capacity in the periphery provides a valid surrogate for the coronary vascular bed. This would potentially facilitate future studies and assist in the identification of pathogenetic mechanisms involved in atherothrombosis. This comparison is the subject of an on-going British Heart Foundation Research Project (PG98150).

11.5 FUTURE DIRECTIONS

In addition to our own work, the exploration of endothelial fibrinolytic dysfunction has been provisionally characterised in patients with hypertension [Hrafnkelsdóttir *et al* 1998], but has not been determined in other conditions associated with atherosclerosis and dysfunction of endothelium-dependent vasomotion. There are many potential candidates that would be appropriate to investigate but three areas have been considered here for discussion.

11.5.1 Renin-Angiotensin System And Endogenous Fibrinolysis

In patients with hypertension, an elevated plasma renin activity for a given urinary sodium excretion is independently associated with an increased risk of acute myocardial infarction [Alderman *et al* 1991]. Moreover, large scale clinical trials in patients with heart failure, ischaemic heart disease and a recent myocardial infarction suggest a reduction in re-infarction rates with angiotensin converting enzyme (ACE) inhibitor therapy. The mechanisms underlying the association of renin-angiotensin system activation with coronary thrombotic events are unknown but may relate, in part, to an effect on fibrinolytic parameters. Indeed, activation of the renin-angiotensin system by sodium depletion causes an increase in early morning plasma PAI-1 concentrations which can be reversed by ACE inhibition [Brown *et al* 1998]. Furthermore, in patients with heart failure [Goodfield *et al* 1999] or a recent myocardial infarction [Wright *et al* 1994; Vaughan *et al* 1997], ACE inhibitor therapy causes marked reductions in basal plasma t-PA and PAI-1 concentrations.

Animal models suggest that bradykinin is an extremely potent stimulus for the release of t-PA from the vascular endothelium *in vitro* [van den Eijnden-Schrauwen *et al* 1995], *ex vivo* [Tranquille & Emeis 1990] and *in vivo* [Schrauwen *et al* 1994]. Studies in man have also indicated that local infusions are associated with elevated levels of t-PA [Brown *et al* 1999; Labinjoh *et al* 2000a]. Bradykinin is not only an inflammatory mediator but is also released during the contact phase of coagulation when high-molecular weight kininogen is cleaved by kallikrein to produce a disulphide-linked light and heavy chain [Schiffman *et al* 1980; Reddigari *et al* 1987]. This liberation of bradykinin may represent an important negative feedback loop in which bradykinin induced t-PA release inhibits thrombus formation within the vascular lumen when localised endothelial denudation occurs. Indeed, in patients with unstable angina, intracoronary thrombus formation is associated with activation of the contact phase of coagulation and the kallikrein system with increased bradykinin generation [Hoffmeister *et al* 1995]. Furthermore, given that bradykinin induced forearm vasodilatation is potentiated by ACE inhibition [Benjamin *et al* 1989], such actions may be enhanced in the presence of ACE inhibition and may, in part, explain the anti-ischaemic action of this therapy [HOPE Investigators 2000]. Recent preliminary data in healthy volunteers have confirmed that ACE inhibition potentiates bradykinin induced t-PA release [Labinjoh *et al* 2000b]. The influence of ACE inhibition on the acute fibrinolytic capacity of the endothelium in patients with ischaemic heart disease and heart failure has yet to be established.

The relationship between the renin-angiotensin system and endogenous fibrinolysis is the subject of an on-going British Heart Foundation Research Project (PG97197).

11.5.2 Hyperhomocysteinaemia And Endogenous Fibrinolysis

Elevated plasma concentrations of homocysteine are an independent risk factor for myocardial infarction [Clarke *et al* 1991; Stampfer *et al* 1992] as well as peripheral and cerebral vascular disease [Kang *et al* 1992]. Severe hyperhomocysteinaemia is rare but moderate hyperhomocysteinaemia is more common [Ueland & Refsum 1989; Kang *et al* 1992; McCully 1996] and present in ~30% of patients with premature coronary artery disease [Clarke *et al* 1991]. Hyperhomocysteinaemia is associated with thrombophilia and a more than 20-fold increased risk of coronary artery disease [Clarke *et al* 1991]. In contrast to serum lipid fractions, the extent of coronary atheroma correlates only weakly with homocysteine concentrations [Nygård *et al* 1997] suggesting that the association with coronary events is more likely to represent thrombogenicity or plaque rupture rather than atherogenicity.

Hyperhomocysteinaemia is a cause of endothelial cell injury and denudation in both animal models [Harker *et al* 1974; Harker *et al* 1976] and human clinical studies [Celermajer *et al* 1993; Tawakol *et al* 1997; Kanani *et al* 1999]. To date, there have been no published studies which have directly addressed the issue of whether there is an association between homocysteine and endogenous fibrinolysis, but it is the subject of much debate [Kuller & Evans 1998]. However, in a longitudinal study of stroke patients, plasma t-PA concentrations were found to be an independent

discriminator and correlated directly with plasma homocysteine concentrations [Lindgren *et al* 1996]. Moreover, methionine loading (a method of acutely elevating plasma homocysteine concentrations) causes more pronounced alterations in basal fibrinolytic parameters of patients with premature vascular disease [Freyburger *et al* 1997]. Thus, it would appear that hyperhomocysteinaemia may be associated with alterations in endogenous fibrinolysis although it is unknown whether this influences the acute release of t-PA from the endothelium.

The relationship between hyperhomocysteinaemia and endogenous fibrinolysis is the subject of an on-going British Heart Foundation Research Project (PG99025).

11.5.3 Inflammation And Endogenous Fibrinolysis

An increased risk of cardiovascular events and death appears to be associated with systemic inflammation and is probably mediated through plaque inflammation, erosion or frank rupture. In epidemiological studies of patients with ischaemic heart disease [Liuzzo *et al* 1994; Haverkate *et al* 1997; Biasucci *et al* 1999; Ferreiros *et al* 1999], and in prospective studies of healthy male populations [Ridker *et al* 1997; Ridker *et al* 2000], serum C-reactive protein and IL-6 concentrations predict the future risk of cardiovascular events. Moreover, the use of anti-inflammatory agents appears to be associated with a reduction in the rate of such events [Ridker *et al* 1997]. Whilst those patients with the largest plaque mass may be expected to have the highest C-reactive protein and IL-6 concentrations, there is evidence that systemic inflammation can enhance the inflammatory activity of the atheromatous

plaque [Libby *et al* 1997; Vallance *et al* 1997] and thereby increase the risk of developing an unstable plaque and an acute coronary syndrome.

Inflammation may have significant direct effects on endothelial function. Recent *in vivo* evidence indicates that inflammatory cytokines induce endothelial vasomotor dysfunction in the dorsal hand veins of man [Bhagat *et al* 1996; Bhagat *et al* 1997]. It is plausible that such dysfunction may result in an impairment of fibrinolytic function and thereby increase the risk of thrombotic and ischaemic events. However, this requires further study and investigation.

The relationship between inflammation and endogenous fibrinolysis is the subject of an on-going British Heart Foundation Junior Research Fellowship (Dr Stanley Chia).

11.6 CLINICAL RELEVANCE

A reduction in t-PA activity or release is associated with an increased incidence of major adverse cardiac events in healthy young men [Meade *et al* 1993], and in patients with stable [Held *et al* 1997] or unstable angina pectoris [Munkvad *et al* 1990; Hoffmeister *et al* 1998]. Moreover, a reduction in fibrinolytic activity in patients with unstable angina is associated with the development of acute myocardial infarction [Munkvad *et al* 1990].

In a recent preliminary study of patients with acute myocardial infarction [Cruden *et al* 2000], we have been able to show that patients who fail to reperfuse with thrombolytic therapy have higher concentrations of plasma t-PA antigen on admission suggesting the presence of t-PA resistant occlusion of the infarct related artery. In contrast, in those patients who reperfuse, lower plasma t-PA activity is associated with a more rapid response to thrombolytic therapy suggesting that patients with impaired endogenous fibrinolysis benefit most from thrombolytic therapy. However, these initial findings need confirmation in a larger cohort of patients and it now needs to be established whether patients with an impaired endogenous fibrinolytic capacity are at greater risk of recurrent cardiac events.

Selective enhancement of local endogenous fibrinolysis could potentially lead to the prevention of thrombotic coronary occlusion and myocardial ischaemic events. Current systemic administration of fibrinolytic agents for myocardial infarction is associated with significant bleeding complications including cerebral and

gastrointestinal haemorrhage. Augmentation of local endothelial t-PA release would be potentially more effective, while avoiding these serious systemic complications.

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APPENDIX

APPENDIX

...tra-arterial substance P mediated vasodilatation in the human forearm: ...armacology, reproducibility and tolerability

...id E. Newby, David G. Sciberras,[1] Carl M. Mendel,[1] Barry J. Gertz,[1] Nicholas A. Boon & David J. Webb

...l Pharmacology Unit and Research Centre, University of Edinburgh, Western General Hospital, Edinburgh and [1]Merck Research Laboratories, ...l Pharmacology—Europe, Terlings Park, Harlow, UK and Rahway, NJ, USA

Aims The current studies were designed to characterize the pharmacology, reproducibility and tolerability of the vasodilator response to intra-arterial substance P infusion in the forearm of healthy man.

Methods On different occasions, eight healthy male volunteers received brachial artery infusions of substance P at doubling doses ranging from 0.5 to 128 pmol min^{-1}. Blood flow was measured in both arms using venous occlusion plethysmography.

Results Substance P induced dose-dependent vasodilatation in the human forearm which had a log-linear relationship to dose. At doses of 1–8 pmol min^{-1}, mean responses were highly reproducible both within and between days. There were no differences between responses to discontinuous doses and continuous doses of substance P. Substance P was generally well tolerated at doses of ≤ 64 pmol min^{-1} with no significant alteration in arterial blood pressure or heart rate. Skin oedema in the infused forearm and systemic vasodilatation, manifested by facial flushing and non-infused forearm vasodilatation, occurred at doses of ≥ 16 pmol min^{-1}.

Conclusions Forearm vasodilatation to substance P represents a reproducible and useful model in the assessment of peripheral endothelial cell NK_1 receptor function.

Keywords: Substance P, vasodilatation, forearm plethysmography, pharmacodynamics, reproducibility, tolerability

...oduction

...stance P is a widely distributed endecapeptide which is ...d principally in the neural tissue of the central, ...pheral and enteric nervous systems [1–4]. The physio... ...al functions of substance P include neurotransmission ...imary sensory neurones, with particular involvement in ...ception and emesis. In addition to functioning as a ...rotransmitter, it also acts as an inflammatory mediator ...] and neurohumoral regulator [4, 8, 9]. Substance P is ...mber of the tachykinin family of peptides and acts ...ugh stimulation of the neurokinin receptors, having a ...ularly high affinity for the type 1 (NK_1) receptor [10]. ...hen given intra-arterially, substance P is a potent ...dilator [11–13] through an endothelium dependent ...hanism [14] which is predominantly mediated by nitric ...de release [15]. This response is induced via stimulation ...e endothelial cell NK_1 receptor [10] and, increasingly, ...stance P is being used to assess endothelial cell function ...alth and disease in man [16–20]. Indeed, substance P ...been infused into the coronary artery of man at doses ...90 pmol min^{-1} [16–18]. However, the reproducibility ...tolerability of intra-arterial substance P in the human ...arm has not been fully characterized and validated. ...ious studies describing such infusions in the forearm ...either used less precise methodology, including unilateral plethysmography and inclusion of the hand circulation [12], or have only incompletely described the nature of the response [13].

Therefore, the aim of the present study was to characterize the pharmacodynamics, reproducibility and tolerability of the vasodilator responses to arterial administration of substance P within the forearm vascular bed of healthy men.

Methods

Subjects

Eight healthy male subjects aged between 20 and 34 years participated in a series of four studies which were undertaken with the approval of the Lothian Research Ethics Committee and the written informed consent of each subject. None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 h and from food, caffeine-containing drinks and tobacco for at least 4 h before each study. All studies were performed in a quiet, temperature controlled room maintained at 23.5–24.5° C.

Drugs

Synthetic pharmaceutical-grade substance P (Clinalfa AG, Läufelfingen, Switzerland) of $\geq 95\%$ purity, was administered following dissolution in physiological saline (0.9% sodium chloride: Baxter Healthcare Ltd, Thetford, UK).

...pondence: Dr D. E. Newby, Clinical Pharmacology Unit and Research Centre, ...sity of Edinburgh, Western General Hospital, Edinburgh EH4 2XU

Intra-arterial administration

The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd, Birmingham, UK) attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, UK) under 1% lignocaine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, UK) local anaesthesia. Patency was maintained by infusion of saline via an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 ml min^{-1}.

Measurements

Blood flow was measured in the infused and non-infused forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges which were applied to the widest part of the forearm [21]. Since forearm length between the occlusion and collecting cuff is constant, volumetric changes are directly proportional to circumferential changes measured by the strain gauge [21, 22]. During measurement periods, the hands were excluded from the circulation by rapid inflation of 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 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 strain gauge Plethysmograph (D.E. Hokanson) was processed by a MacLab® analogue-to-digital converter and Chart® v3.3.8 software (AD Instruments Ltd, Castle Hill, Australia) and recorded onto a MacIntosh Classic II computer (Apple Computers Inc, Cupertino, USA). Calibration was achieved using the internal standard of the plethysmograph.

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) [23].

Study design (Figure 1)

Subjects rested recumbent throughout each study. Strain gauges and cuffs were applied, and the brachial artery of the

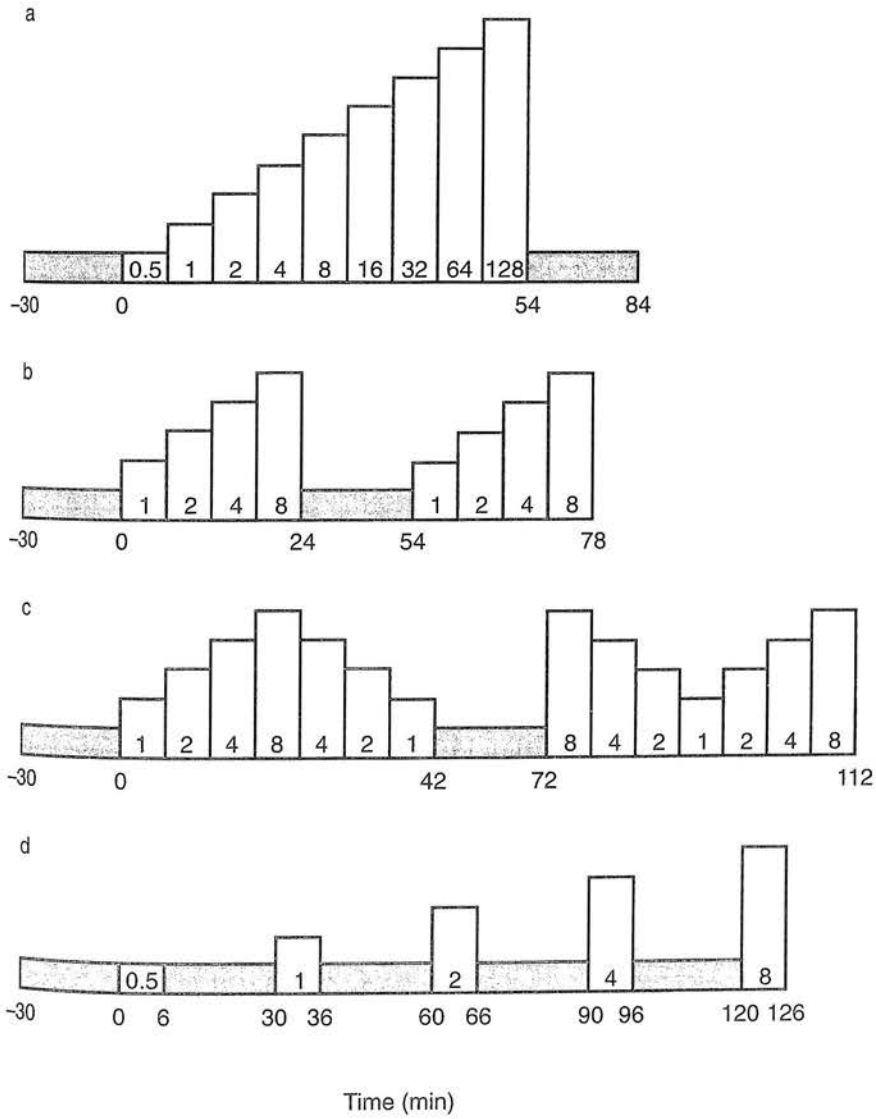

Figure 1 Study design: protocols a, b, c and d with saline (▨) and substance P (pmol min^{-1}) (□) infusions.

...dominant arm cannulated. Saline was infused for the ...30 min to allow for equilibration. Forearm blood flow ... measured for 3 min beginning at 25, 15 and 6 min ...re commencing substance P infusions. Throughout all ...ds, substance P was infused for 6 min at each dose. ...arm blood flow measurements were made for the last ...n of each infusion period.

...he tolerability of substance P was assessed in seven ...cts who were given incremental doubling doses of ...tance P from 0.5 pmol min^{-1} to a maximum of ...pmol min^{-1} [12], followed by 30 min infusion of saline ...tocol a). To determine within and between day ...ducibility, each subject reattended 7 to 14 days later ...received 1, 2, 4 and 8 pmol min^{-1} of substance P ...wed by saline for 30 min before receiving further ...mental doses of 1, 2, 4 and 8 pmol min^{-1} of substance P ...tocol b).

...order to examine whether responses to substance P ...ibit tachyphylaxis, seven subjects were given substance P ...sions at sequential doses of 1, 2, 4, 8, 4, 2 and 1 pmol ...$^{-1}$, followed by saline for 30 min before receiving ...tance P at sequential doses of 8, 4, 2, 1, 2, 4 and 8 pmol ...$^{-1}$ (protocol c). Seven to 14 days later, each subject ...anded and received an infusion of substance P at doses ...5, 1, 2, 4 and 8 pmol min^{-1} with each dose separated ...19 min period of saline infusion (protocol d).

...he subject withdrew for personal reasons after complet- ...protocols a and b, and was replaced for protocols c and d.

...analysis and statistics

...plethysmographic data were extracted from the Chart data ...and forearm blood flows were calculated for individual ...us occlusion cuff inflations by use of a template ...sheet (Excel v4.0; Microsoft Inc, USA). Recordings ...the first 60 s after wrist cuff inflation were not used, ...se of the instability in blood flow that this causes [24]. ...ly, the last five flows recorded in each 3 min measure- ...period were calculated and averaged for each arm. Basal ...flow was taken to be that recorded at 6 min before ...infusion. To reduce the variability of blood flow data, ...tio of flows in the two arms was calculated for each ...point, in effect using the non-infused arm as a ...mporaneous control for the infused arm [21].

...ta were examined by two way analysis of variance ...OVA) with repeated measures, regression analysis and two tailed Student's *t*-test using Excel v4.0 (Microsoft). All results are expressed as means ± s.e. mean. Statistical significance was taken at the 5% level. Within and between day reproducibility was assessed using the method of Bland & Altman [25] and coefficients of repeatability were determined for 95% confidence intervals using the Student's *t* distribution.

Results

Tolerability of intra-arterial substance P infusion

Transient and patchy flushing of the infused arm occurred at all doses, whereas facial flushing was only observed at doses of $\geq$32 pmol min^{-1}. Five subjects received 64 pmol min^{-1} and two subjects received 128 pmol min^{-1} as the maximal dose: further infusion of substance P was discontinued because of extensive forearm skin oedema and facial flushing. There were no significant increases in heart rate or decreases in blood pressure up to 64 pmol min^{-1} (Table 1). No subject reported discomfort or local forearm fullness with substance P infusion, but two subjects described transient light-headedness at doses of 16 and 64 pmol min^{-1}. In the infused forearm, patchy skin oedema developed in some subjects at 16 pmol min^{-1} and consistently, in all by 32 pmol min^{-1}. The oedema had an urticarial appearance, taking the form of a raised wheal with a yellow hue. However, there was no associated pruritis and the lesions were non-tender. The extent of oedema varied between subjects, beginning at the level of the elbow and extending distally with increasing dose. The affected areas ranged from 1 to 10 cm in diameter, but all resolved completely within 1–2 h of stopping the infusion.

Blood flow responses

Dose range of vasodilator responses to substance P Substance P increased blood flow in the infused forearm ($P<0.001$) in a dose-dependent manner which reached a maximum of 21 ± 3.1 ml 100 ml^{-1} min^{-1} (466 ± 192%) by 16 pmol min^{-1} (Figure 2 and Table 2). There was a significant increase in the blood flow of the contralateral, non-infused arm ($P=0.001$, ANOVA) which was apparent from 16 pmol min^{-1} ($P=0.05$, *t*-test). Relative percentage increase in blood flow of the infused to non-infused arm was dose-dependent and peaked at 32 pmol min^{-1} before declining at 64 pmol min^{-1}. The increases in blood flow had a linear

...1 Systemic haemodynamics and blood flow responses (mean ± s.e.mean) to incremental doses of substance P (*$P=0.05$; ...005 *vs* baseline).

			Substance P infusion (pmol min^{-1})		
		Baseline	*2*	*16*	*64*
...ressure	Systolic	140 ± 6	139 ± 4	139 ± 4	138 ± 7
...Hg)	Diastolic	70 ± 4	68 ± 5	69 ± 6	65 ± 4
...te (beats min^{-1})		57 ± 4	56 ± 4	61 ± 3	63 ± 3
...low	Infused arm	3.7 ± 0.7	11.7 ± 1.5§	20.9 ± 2.8§	21.5 ± 2.4§
...00 ml^{-1} min^{-1})	Non-infused arm	3.4 ± 0.4	3.6 ± 0.5	4.6 ± 0.9*	8.8 ± 1.7§
...se in forearm blood ...elative to non-infused arm		–	233 ± 59%§	466 ± 197%§	221 ± 105%§

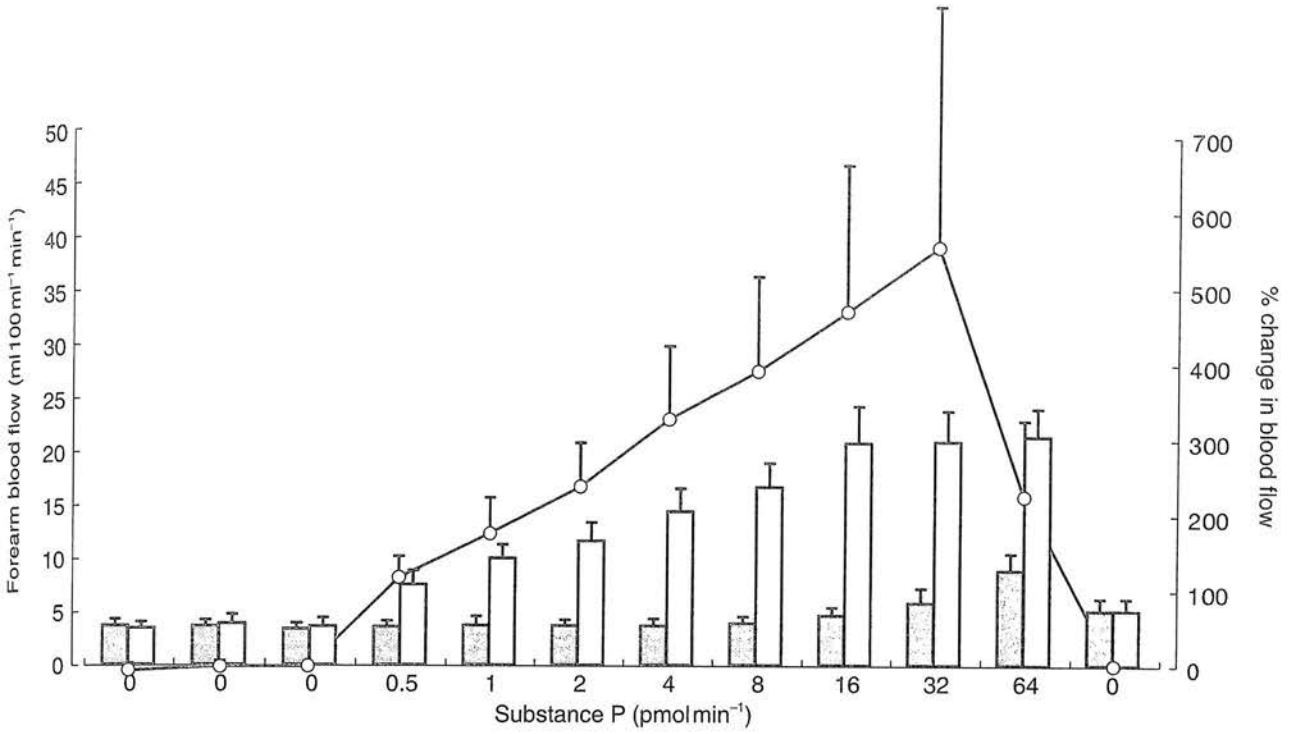

Figure 2 Absolute and relative forearm blood flow responses to incremental doses of substance P (protocol a). (Mean ± s.e.mean). ▨ non-infused arm, □ infused arm, ○ % change infused/non-infused.

Table 2 Absolute forearm blood flow (ml 100 ml^{-1} of tissue min^{-1}) in both arms during the four protocols (mean ± s.e.mean).

Protocol a	*Substance P infusion (pmol min^{-1})*									
	0	*0.5*	*1*	*2*	*4*	*8*	*16*	*32*	*64*	*0*
Infused arm	4.1 ± 0.7	7.9 ± 1.2	10.0 ± 1.5	11.7 ± 1.8	14.6 ± 2.4	16.5 ± 2.6	19.3 ± 3.1	19.7 ± 3.0	21.4 ± 2.9	5.7 ± 1.2
Non-infused arm	3.7 ± 0.4	4.0 ± 0.4	4.1 ± 0.5	3.9 ± 0.4	4.2 ± 0.6	4.3 ± 0.6	5.0 ± 0.9	6.7 ± 1.3	9.8 ± 1.6	5.7 ± 1.1
Protocol b	*Substance P infusion (pmol min^{-1})*									
	0	*1*	*2*	*4*	*8*	*0*	*1*	*2*	*4*	*8*
Infused arm	4.8 ± 0.9	9.5 ± 1.7	11.1 ± 1.8	14.4 ± 2.3	17.5 ± 2.6	5.5 ± 1.2	9.8 ± 1.8	11.0 ± 1.7	15.2 ± 2.7	18.3 ± 3.7
Non-infused arm	4.1 ± 0.4	4.2 ± 0.5	4.1 ± 0.5	4.3 ± 0.5	4.5 ± 0.6	6.0 ± 1.1	5.9 ± 1.0	5.4 ± 0.8	5.6 ± 1.0	5.4 ± 0.8
Protocol c	*Substance P infusion (pmol min^{-1})*									
	0	*1*	*2*	*4*	*8*	*4*	*2*	*1*	*0*	
Infused arm	3.4 ± 0.5	9.5 ± 0.8	11.3 ± 1.2	13.1 ± 1.4	17.5 ± 1.9	12.6 ± 1.3	10.4 ± 1.1	8.1 ± 0.9	4.2 ± 0.5	
Non-infused arm	3.2 ± 0.4	3.2 ± 0.4	3.1 ± 0.5	3.0 ± 0.4	2.9 ± 0.4	3.2 ± 0.4	3.3 ± 0.4	3.2 ± 0.4	3.3 ± 0.4	
	Substance P infusion (pmol min^{-1})									
	0	*8*	*4*	*2*	*1*	*2*	*4*	*8*		
Infused arm	3.7 ± 0.5	19.6 ± 1.7	12.4 ± 1.2	10.1 ± 0.9	7.3 ± 0.4	9.7 ± 0.8	11.6 ± 0.9	17.2 ± 1.7		
Non-infused arm	3.9 ± 0.4	3.7 ± 0.6	3.3 ± 0.4	3.4 ± 0.5	3.3 ± 0.4	3.3 ± 0.3	3.5 ± 0.4	3.5 ± 0.5		
Protocol d	*Substance P infusion (pmol min^{-1})*									
	0	*0.5*	*0*	*1*	*0*	*2*	*0*	*4*	*0*	*8*
Infused arm	3.4 ± 0.6	7.5 ± 0.6	3.9 ± 0.8	10.7 ± 0.9	4.3 ± 0.7	15.0 ± 1.7	4.4 ± 0.7	18.9 ± 3.1	4.4 ± 0.8	25.3 ± 3.5
Non-infused arm	3.5 ± 0.6	3.4 ± 0.6	4.0 ± 0.7	3.9 ± 0.6	4.2 ± 0.6	4.1 ± 0.8	4.4 ± 0.7	4.6 ± 0.9	4.7 ± 0.8	4.7 ± 0.8

relationship to the logarithm of substance P dose (Figure 2: $y = 174 + 242x$; $r = 0.997$, $P < 0.001$) at doses ≤ 32 pmol min^{-1}.

Reproducibility of vasodilator responses to substance P There were no significant differences between the mean vasodilator responses to substance P either between days or within a

day (Figure 3a). The reproducibility of individual in day and between day responses are shown in Table 3. 95% confidence intervals indicated by the coefficients repeatability, are ~2–4 fold smaller for within day ponses compared to those between day. These data, for mple population of eight subjects at $P<0.05$, give 95% er to detect a mean shift in the dose response of >4 and >8 fold when comparing within day and between responses respectively.

There was a trend for the magnitude of mean vasodilator ponses to substance P to undergo attenuation with tinuous infusions, but this did not achieve statistical ficance (Figure 3b). Likewise, there were no significant erences between the vasodilatation to isolated discontinu- doses and incremental continuous doses of substance P ure 3c). Absolute forearm blood flows in both arms in ur protocols are shown in Table 2.

ussion

-arterial substance P infusion at doses up to and luding 64 pmol min^{-1} were generally well tolerated with gnificant changes in supine blood pressure and heart . Previous studies [12, 26, 27] using intravenous dosing induced flushing, tachycardia and decreased blood ure at higher doses (>150 pmol min^{-1}). However, oedema associated with arterial infusion has not been ted before, despite intra-brachial infusions of up to mol min^{-1} [12]. This disparity may reflect the iously shorter infusion times of 4 min per dose [12] the relative purities of substance P administered. rtheless, our observations are consistent with the action bstance P as an inflammatory mediator inducing protein rvasation and leucocyte migration [6]. These effects may t baseline forearm circumference but not the rate of rm expansion with plethysmographic measurements s the rate of oedema formation were to approach that rearm blood flow, or oedema formation were to raise extracellular fluid pressure above 40 mmHg. This ld be associated with substantial tissue swelling and ld exceed the observed limited oedematous response.

bstance P has been used as an endothelium dependent dilator in the human coronary circulation [16–18]. Total nary blood flow is ~300 ml min^{-1} (60–90 ml l^{-1} of tissue min^{-1}) which compares with ml min^{-1} (3–5 ml 100 ml^{-1} of tissue min^{-1}) in the rm. Although left coronary artery blood flow is, fore, ~3–4 times higher than that in the brachial , substance P does increase left coronary blood flow and at concentrations similar to those achieved in the forearm [18]. Therefore, given that substance P can cause oedema in the forearm and the consequences of unrecognised myocardial oedema may be serious, we would caution against the intra-coronary administration of high doses of substance P in man.

Intra-arterial substance P caused consistent and dose-dependent local increases in forearm blood flow without systemic effects at doses up to and including 8 pmol min^{-1}. In agreement with previous studies [12, 13, 15, 19, 20, 28], the vasodilator response was linearly related to the logarithm of the substance P dose. However, at doses of greater than or equal to 16 pmol min^{-1}, substance P induced systemic vasodilatation as indicated by facial flushing and an increase in forearm blood flow of the contralateral non-infused arm, although no significant decrease in blood pressure, or increase in heart rate, was observed. The use of comparative increases in forearm blood flow of the infused forearm with respect to the non-infused arm, becomes invalid at systemic doses and accounts for the breakdown of the log-linearity of responses at doses of more than 32 pmol min^{-1}.

McEwan and colleagues [13] have shown that substance P at 1 pmol min^{-1} undergoes tachyphylaxis when infused continuously for 10 min or more. However, we did not find a statistically significant attenuation of the responses when administering decremental doses of substance P. Moreover, we found no greater response with incremental discontinuous infusions than with incremental continuous doses of substance P. Mean increases in forearm blood flow to substance P at doses of 1 to 8 pmol min^{-1} were of equivalent magnitude both within day and between days. Eklund and colleagues [12] have reported variable measurements with substance P, but their results are confounded by utilising cruder methodology with unilateral forearm plethysmography and inclusion of the hand circulation. In contrast to the muscular forearm, the hand is predominantly skin and has a heterogeneous circulation which is regulated in a complex and non-linear manner [21]. We have demonstrated good reproducibility when comparing individual within day responses. Based on a sample size of eight, the confidence intervals would indicate that we would have 95% power to detect >4-fold shift in the dose-response relationship when comparing within day responses at each dose.

In summary, we have found using within day comparisons, that at doses up to 8 pmol min^{-1}, vasodilatation to substance P is generally well tolerated and highly reproducible. Such methodology should provide a practical and sensitive method of assessing the *in vivo* efficacy of peripheral NK_1 receptor antagonism in man [29].

e 3 Within and between day reproducibility for individual percentage increases in forearm blood flow at 1, 2, 4 and 8 pmol min^{-1}.

P dose min^{-1})	Within day responses			Between day responses		
	Mean % change in blood flow	*Mean of the differences*	*Coefficient of repeatability*	*Mean % change in blood flow*	*Mean of the differences*	*Coefficient of repeatability*
	131	24.4	32	158	30.7	61
	189	24.7	49	217	31.3	74
	281	−35.5	34	292	57.6	144
	362	−22.9	30	368	34	127

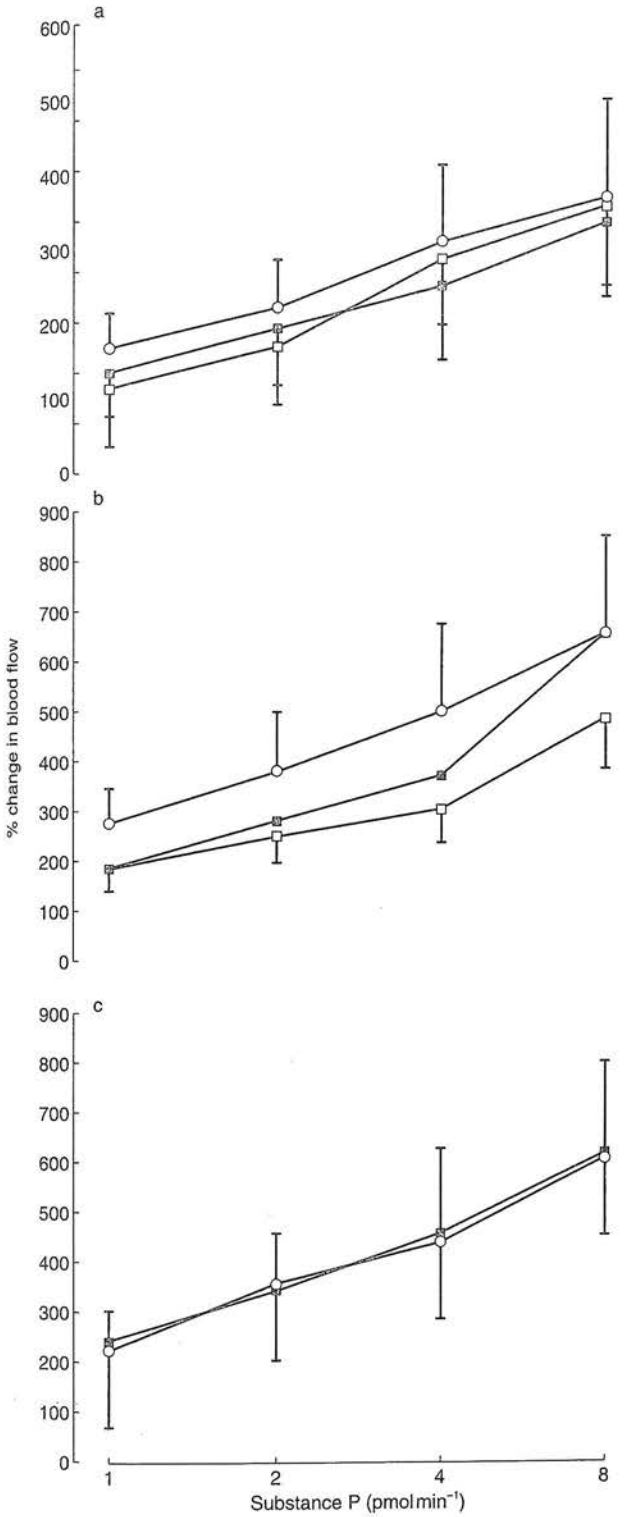

Figure 3 a) Within and between day mean increases in forearm blood flow to 1, 2, 4 and 8 pmol min^{-1}: protocol a dose response (○); protocol b, first dose response (▩); and protocol b, second dose response (□). b) Development of tachyphylaxis with protocol c: first dose response (○); second and third dose responses (▩); both were superimposable and the second response only is shown for clarity); and fourth dose response (□). c) Comparison of isolated discontinuous and incremental continuous infusions: protocol c, first dose response (▩); and protocol d, first dose response (○). (mean ± s.e.mean).

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Substance P-induced vasodilatation is mediated by the neurokinin type 1 receptor but does not contribute to basal vascular tone in man

David E. Newby, David G. Sciberras,[1] Charles J. Ferro, Barry J. Gertz,[1] David Sommerville, Anup Majumdar,[1] Richard C. Lowry[1] & David J. Webb

Clinical Pharmacology Unit and Research Centre, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh, Scotland, UK and [1]Merck Research Laboratories, Clinical Pharmacology—Europe, Terlings Park, Harlow, UK, and Rahway, NJ, USA

Aims Following intravenous administration of its prodrug, L-758,298, we assessed the pharmacodynamics of L-754,030, a novel and highly selective NK_1 receptor antagonist, by examining systemic haemodynamics and the blood flow responses to intra-arterial substance P infusion.

Methods Sixteen healthy male volunteers participated in a double-blind, randomised, placebo controlled crossover trial of L-758 298. Forearm blood flow was measured using venous occlusion plethysmography during intrabrachial substance P infusion (0.125–128 pmol min^{-1}). In part 1, eight subjects received substance P infusions before and during placebo, 0.25 mg, 1 mg or 5 mg of L-758 298. In part 2, eight subjects received substance P infusions 24 h after placebo or 1.43 mg of L-758 298.

Results L-758 298 caused dose dependent inhibition of substance P induced vasodilatation ($P<0.001$). Placebo adjusted differences (95% CI) in baseline forearm blood flow, mean arterial pressure and heart rate showed no relevant changes with 5 mg of L-758 298 (>1400–fold shift in substance P response): 0.00 (−0.49 to +0.49) ml 100 ml^{-1} min^{-1}, 1.0 (−3.2 to +5.2) mmHg and 1.9 (−5.9 to +9.7) beats min^{-1}, respectively. Twenty-four hours after 1.43 mg of L-758,298, there was ~34–fold shift in response to substance P induced vasodilatation ($P<0.008$) at plasma L-754 030 concentrations of 2–3 ng ml^{-1}. L-758 298 was generally well tolerated without serious adverse events.

Conclusions Substance P induced forearm vasodilatation is mediated by the endothelial cell NK_1 receptor in man but endogenous substance P does not appear to contribute to the maintenance of peripheral vascular tone or systemic blood pressure.

Keywords: substance P, neurokinin 1 receptor, endothelium, haemodynamics, blood flow

Introduction

Substance P is a widely distributed endecapeptide which is found principally in the neural tissue of the central, peripheral and enteric nervous systems [1–4]. The physiological functions of substance P include neurotransmission in primary sensory neurones with particular involvement in nociception and emesis. In addition to functioning as a neurotransmitter, it also acts as an inflammatory mediator [5–7] and neurohumoral regulator [1, 8].

Correspondence: Dr D. E. Newby, Clinical Pharmacology Unit and Research Centre, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, Scotland, UK. Tel.: 0131–332 1205, Facsimile: 0131–343 6017, E-mail: D. E. Newby@ed.ac.uk.

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Substance P is a member of the tachykinin family of peptides and acts through stimulation of the neurokinin receptors, having a particularly high affinity for the type 1 (NK_1) receptor [9]. When given intra-arterially, substance P is a potent vasodilator [10–12] through an endothelium dependent mechanism [13] which is partly mediated by nitric oxide release [14, 15]. In animal studies, this response is induced via stimulation of the endothelial cell NK_1 receptor [9] although, to date, this has not been confirmed *in vivo* in man. Substance P is found in perivascular neural tissue [16] and has been postulated to play a role in the regulation of vascular tone [17, 18].

Antagonism of the NK_1 receptor has potentially diverse therapeutic indications such as in the treatment of pain, inflammation and emesis [19]. L-754 030 (2-(R)-(1-(R)

-3,5-bis (trifluoromethyl) phenylethoxy)-3-(S)-(4-fluoro) phenyl-4-(3-(5-oxo-4H-1,2,4-triazolo)methyl morpholine; also known as MK-869) is a long acting, highly selective, competitive NK_1 receptor antagonist with poor solubility in aqueous solution. L-754 030 is more selective for the NK_1 than the NK_3 (3000–fold) or the NK_2 and other G-protein linked receptors and ion channels (>50 000–fold) [20]. N-phosphorylation of L-754 030 produces L-758 298, a prodrug which is readily soluble in aqueous solutions. L-758 298 undergoes rapid *in vivo* conversion to L-754 030 and thereby provides a prodrug which can be administered intravenously.

The primary aims of the present study were: first, to determine the ability of L-754 030 to inhibit substance P induced vasodilatation during and 24 h after intravenous administration of L-758 298; second, to confirm that substance P induced vasodilatation is mediated via the endothelial cell NK_1 receptor in man; and third, to determine whether endogenous substance P regulates peripheral vascular tone or blood pressure in man. An additional important aim of the study was to evaluate the tolerability of single intravenous doses of L-758 298 in healthy male volunteers.

Methods

Subjects

Healthy nonsmoking men aged between 18 and 45 years participated in a series of studies which were undertaken with the approval of the Lothian Research Ethics Committee and the written informed consent of each subject. None of the subjects was taking regular medications, or received vasoactive or nonsteroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 h and from food and caffeine-containing drinks for at least 9 h before each study. All studies were performed in a quiet, temperature controlled room maintained at 23.5–24.5° C.

Drug administration

The brachial artery of the nondominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under 1% 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 P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 ml min^{-1}.

Synthetic pharmaceutical-grade substance P (Clinalfa AG, Läufelfingen, Switzerland) of ≥95% purity, was administered following dissolution in saline.

Matched placebo and L-758 298 (Merck Research Laboratories, West Point, USA) were reconstituted with 0.9% saline in glass vials containing 50 mg of mannitol alone or 6 mg of L-758 298 and 50 mg of mannitol, respectively. The 5 mg initial dose of L-758 298 was chosen to exceed the ID_{90} of 50 $\mu g\ kg^{-1}$ in the guinea pig sensorotoxin-induced systemic vascular leak model (Merck Research Laboratories, Terlings Park, UK). Cannulae were inserted into the veins of the antecubital fossae of both arms. L-758 298 was administered into the dominant arm via a 19-G cannula (Wallace Y-Can; Wallace Ltd, Colchester, UK) and venous samples were withdrawn from the nondominant arm via a 17-G cannula (Venflon; BOC Ohmeda AB, Helsingborg, Sweden).

Measurements

Blood flow was measured in the dominant and nondominant forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges which were applied to the widest part of the forearm [21]. Since forearm length between the occlusion and collecting cuff is constant, volumetric changes are directly proportional to circumferential changes measured by the strain gauge [21, 22]. During measurement periods, the hands were excluded from the circulation by rapid inflation of 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 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 strain gauge Plethysmograph (D. E. Hokanson) was processed by a MacLab® analogue-to-digital converter and Chart® v3.3.8 software (AD Instruments Ltd, Castle Hill, Australia) and recorded onto a MacIntosh Classic II computer (Apple Computers Inc, Cupertino, USA). Calibration was achieved using the internal standard of the plethysmograph.

Blood pressure was monitored in the dominant arm at intervals throughout each study using a semiautomated noninvasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan) [23].

Blood (10 ml) was withdrawn from the nondominant arm before and after the incremental infusion of substance P and admixed with 1 ml of 1% disodium EDTA. Blood samples were placed on ice before being centrifuged at 2000 *g* for 30 min. Plasma was frozen and stored at −80° C prior to assay. Plasma L-754 030 concentrations were determined using high performance liquid chromatography and mass spectrometry using an internal standard

(L-752 611). The assay was validated over a concentration range of 1–500 ng ml^{-1} with a limit of detection at 1 ng ml^{-1} and a coefficient of variation of <9%.

Tolerability assessments were made before, during and following completion of the study and included: clinical examination, repeated questioning for symptoms, clinical chemistry screen (liver enzymes, bilirubin, electrolytes, urea, creatinine, protein and albumin), haematology screen (full blood and differential count), urinalysis, 12-lead electrocardiogram.

Study design (Figure 1)

Subjects attended at 09.00 h, rested recumbent throughout each study and intravenous cannulae were inserted into each arm. Strain gauges and cuffs were applied, and the brachial artery of the nondominant arm cannulated. Saline was infused into the arterial cannula for the first 30 min to allow for equilibration. Forearm blood flow was measured for 3 min beginning at 23, 13 and 3 min before commencing substance P infusions. Throughout all studies, substance P was infused for 10 min at each dose. Forearm blood flow measurements were made from 3 to 6 min of each infusion period.

Screening Before inclusion in the main study, subjects received intra-arterial infusions of substance P at 0.125, 0.5, 2, 8 and 32 pmol min^{-1} [24]. To reduce overall variability in the responses, subjects were recruited to the main study if 2 pmol min^{-1} of substance P increased forearm blood flow by ≥100% and ≤500%.

Part 1—Maintenance L-758 298 infusion On each occasion, eight subjects received incremental intra-arterial infusions of substance P at 0.125, 0.5, 2 and 8 pmol min^{-1} followed by 60 min of saline. A second infusion of substance P was then administered at 0.125, 0.5, 2, 8, 32 and 128 pmol min^{-1} [24].

At the beginning of the intervening 60 min saline infusion, subjects received a double-blind, randomised intravenous infusion of either L-758 298 or placebo: (Table 1). An intravenous bolus (two-thirds of the total

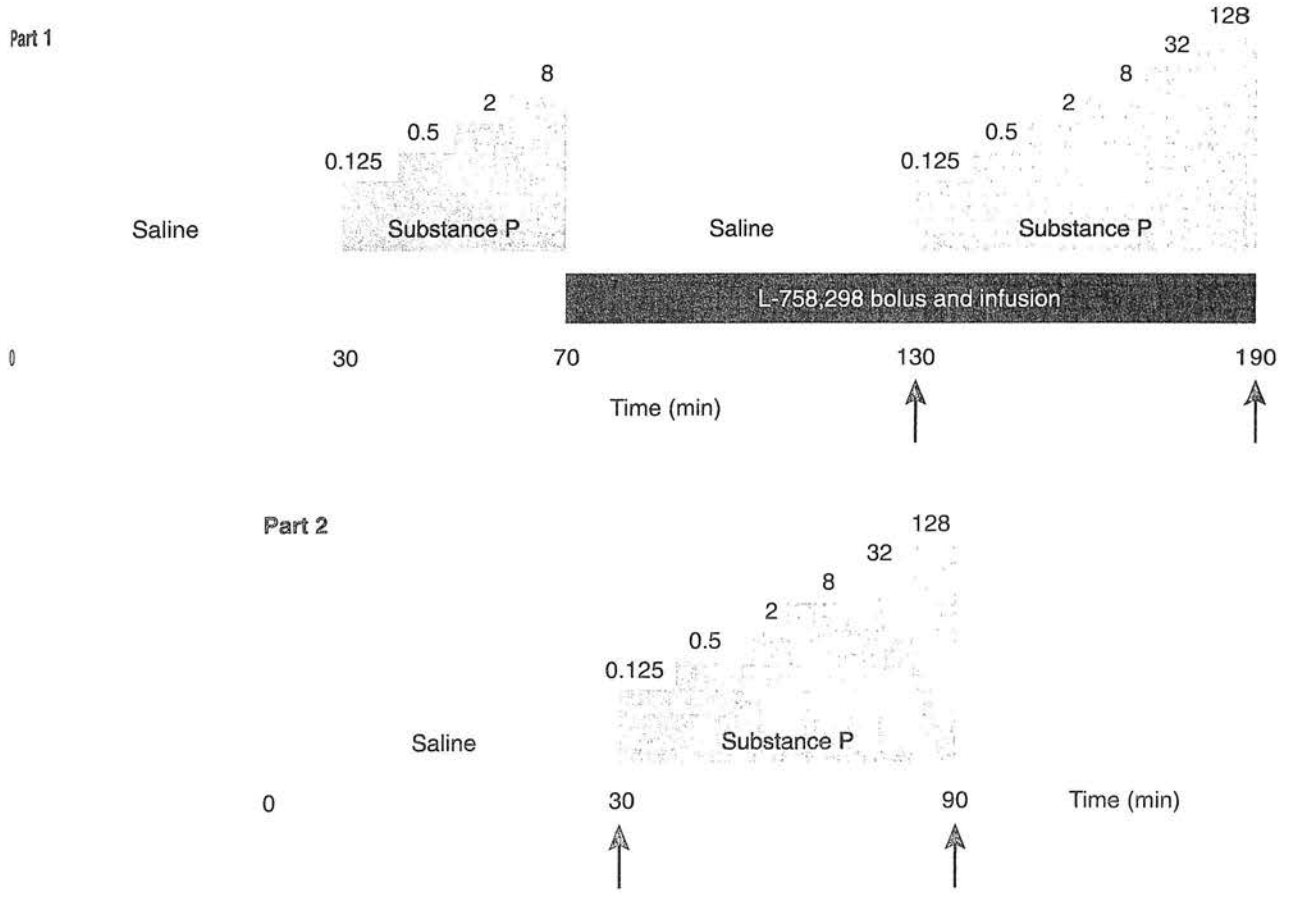

Figure 1 Schematic of protocol. Doses of substance P given in pmol min^{-1}. Arrows indicate blood sampling time points for the estimation of plasma L-754 030 concentrations. Part 2 infusions were commenced 24 h after 1.43 mg of intravenous L-758 298 administration.

Table 1 Schematic of drug allocation.

Subject	Week 1	Week 2*	Week 3†	Week 4
1	Placebo	D_1	D_2	D_3
2	D_1	Placebo	D_2	D_3
3	D_1	D_2	Placebo	D_3
4	D_1	D_2	D_3	Placebo
5	Placebo	D_1	D_2	D_3
6	D_1	Placebo	D_2	D_3
7	D_1	D_2	Placebo	D_3
8	D_1	D_2	D_3	Placebo

D_1=5 mg of L-758 298. *If the PD_{100} increases by >40-fold then D_2=1 mg of L-758 298; if <40-fold, D_2=20 mg. †If the PD_{100} continues to be >40-fold then D_3=0.25 mg of L-758 298 or <40-fold, D_3=80 mg: otherwise the study is completed after 3 weeks i.e. D_3=placebo.

dose over 20 min) was given followed by a continuous maintenance infusion (one third of the total dose over the subsequent 100 min) throughout the second challenge of intra-arterial substance P administration. From earlier phase I pharmacokinetic studies in man (Merck; data on file), this dosage regimen was predicted to produce a stable plasma concentration of L-754 030 during the second incremental infusion of intra-arterial substance P. Plasma samples to measure L-754 030 concentrations were taken 60 and 120 min after the start of the intravenous infusion of L-758 298 or placebo. In the first week, subjects received a total dose of 5 mg of L-758 298 or placebo. The next dose administered in the second week would be increased to 20 mg if the PD_{100} (defined below) was increased by <40-fold by 5 mg of L-758 298 or reduced to 1 mg if >40-fold (based on data from 6 subjects). The same criteria were applied to determine whether the final dose (80 mg or 0.25 mg) of L-758 298 was to be administered, or the study completed after two doses of L-758 298 and placebo (based on data from four subjects; see Table 1). If the third dose was not to be given then this would be replaced by placebo and the study terminated after 3 weeks. To maintain double-blind randomization, all data analysis and dosage decisions were made at the central co-ordinating centre (Merck Research Laboratories, Terlings Park, UK) independent of the investigators.

Part 2–24 h post L-758 298 infusion Eight further subjects attended on two occasions, 1 week apart, and received an intravenous infusion of either L-758 298 or placebo over 30 min in a double-blind randomised manner. Subjects returned 24 h later to receive infusions of intra-arterial substance P at 0.125, 0.5, 2, 8, 32 and 128 pmol min^{-1} [24]. Plasma samples to measure L-754 030 concentrations were taken 24 and 25 h after the start of the intravenous infusion of L-758 298 or placebo. The dose of L-758 298 was derived from the pharmacodynamic and pharmacokinetic data of part 1 and was chosen to produce an approximately 40–fold increase in the PD_{100} 24 h after L-758 298 administration. L-754 030 has a plasma half-life of 15 ± 4 h in man.

Data analysis and statistics

Plethysmographic data were extracted from the Chart data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel v5.0; Microsoft Inc, USA). Recordings from the first 60 s after wrist cuff inflation were not used, because of the instability in blood flow that this causes [25]. Usually, the last five flows recorded in each 3 min measurement period were calculated and averaged for each arm. Basal blood flow was taken to be that recorded immediately before drug infusion. To reduce the variability of substance P blood flow responses, the ratio of flows in the two arms was calculated for each time point, in effect using the dominant arm as a contemporaneous control for the nondominant arm [21]. Plethysmographic data were compiled by blinded investigators.

Percentage change in forearm blood flow from baseline was calculated using the ratios of flows in the two arms for each of the substance P infusions. Natural logarithm transformations of the substance P doses were used to estimate individual PD_{100} values using linear regression techniques. The PD_{100} was defined as the interpolated or extrapolated dose of substance P which provokes a 100% increase in forearm blood flow. To determine whether there was a within day difference in PD_{100} following placebo, an analysis of variance (ANOVA) with the terms 'subjects' and 'time' (predose and postdose) was used to analyse the natural logarithm transformed PD_{100} data from the placebo treatment group in part 1.

The influence of each dose of L-758 298 in parts 1 and 2 on the forearm blood flow response to substance P were evaluated relative to placebo. In these analyses, the PD_{100} after administration of a placebo dose was used as the control for assessment of the fold shifts in PD_{100} due to the administration of the various doses of L-758 298. An ANOVA with the terms 'subject' and 'treatment' was used to analyse the natural logarithm transformed PD_{100} data from the treatments in part 1. An ANOVA appropriate for a two period crossover study was used to analyse the natural logarithm transformed PD_{100} data from the treatments in part 2. These ANOVA analyses were used to estimate the geometric means, their ratios and 95% confidence intervals for the geometric mean ratio for PD_{100}.

Mean arterial pressure, heart rate and blood flow data

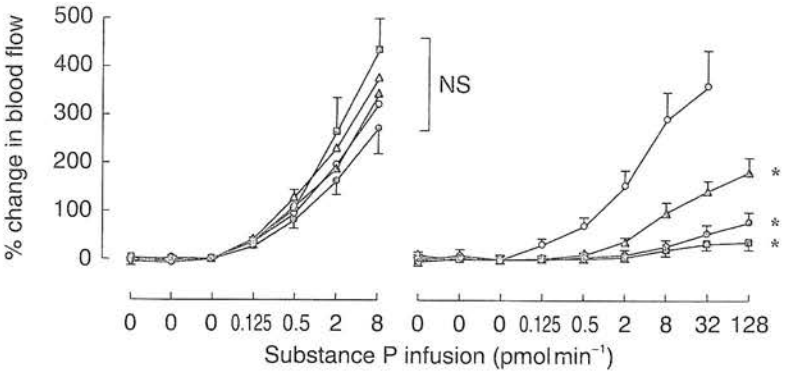

Figure 2 Part 1: Forearm blood flow responses to intrabrachial substance P infusion before (left panel) and during placebo or L-758 298 infusion (right panel). Mean ± s.e.mean. ○ Placebo; ▲ 0.25 mg, ⊕ 1 mg and ▩ 5 mg of L-758 298. *$P<0.001$; L-758 298 *vs* placebo, ANOVA.

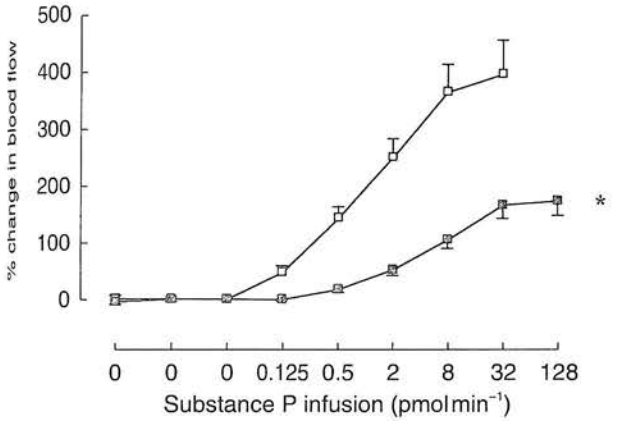

Figure 3 Part 2: Forearm blood flow responses to intrabrachial substance P infusion. (Mean ± s.e.mean.) Placebo; 1.43 mg of L-758,298* $P<0.001$; L-758 298 *vs* placebo, ANOVA.

were examined, where appropriate, by multifactorial ANOVA with repeated measures and paired Student's *t*-test. All results are expressed as mean ± s.e. mean. Statistical significance was taken at the 5% level.

Results

Twenty-two healthy male volunteers were required to identify 16 subjects who met the entry criteria: forearm blood flow increased by >500% in two subjects and <100% in four subjects. The 16 volunteers subsequently recruited were aged 30 ± 2 years (range 20–40 years) and weighed 76 ± 3 kg (range 61–95 kg).

There were no significant baseline differences in forearm blood flow, blood pressure or heart rate between any parts of the study.

Part 1

Substance P caused dose-dependent vasodilatation in the nondominant arm ($P<0.001$; ANOVA) during the first incremental infusion of substance P which was not significantly different between screening or the 4 separate study days.

L-758 298 was administered in three descending doses: 5, 1 and 0.25 mg. During placebo and L-758 298 infusion, there were no significant changes in blood pressure, heart rate, or blood flow in the dominant forearm (Table 2). Placebo adjusted differences (95% CI) in dominant forearm blood flow, mean arterial pressure and heart rate with 5 mg of L-758 298 were 0.00 (−0.49–0.49) ml 100 ml^{-1} min^{-1}, 1.0 (−3.2–5.2) mmHg and 1.9 (−5.9–9.7) beats min^{-1}, respectively. Plasma concentrations of L-754 030 were not significantly different immediately before and after the second incremental infusion of substance P (Table 2).

In comparison with the response to the first incremental infusion, the response to the second infusion of substance P was significantly different following placebo ($P<0.006$) and all doses of L-758 298 in a dose dependent manner (Table 2; Figure 2)). The geometric mean PD_{100} (95% CI) increased by 1.85-fold (1.27–2.86) during placebo infusion. The influence of each dose of L-758 298 on the forearm blood flow response to substance P was examined relative to placebo. The geometric mean PD_{100} (95% CI) increased by 30–fold (9–99) with 0.25 mg, 319–fold (98–1044) with 1 mg and >1400–fold with 5 mg of L-758 298 ($P<0.001$ for all).

Forearm blood flow in the dominant arm increased at doses ≥32 pmol min^{-1} of substance P only during placebo ($P<0.02$ *vs* baseline; paired *t*-test). Heart rate and blood pressure did not change significantly (Table 2).

Part 2

Following placebo infusion, substance P again caused dose-dependent vasodilatation in the nondominant arm ($P<0.001$; ANOVA: Figure 3). However, 24 h following intravenous administration of 1.43 mg of L-758 298,

Table 2 Mean arterial pressure (MAP), heart rate (HR), forearm blood flow and plasma L-754 030 concentrations during substance P challenges in parts 1 and 2 (see Figure 1). Mean ± s.e.mean.

	Baseline					Maximum substance P dose§				
Study protocol	*MAP (mmHg)*	*HR (beats min^{-1})*	*Non-dominant arm blood flow (ml 100 ml^{-1}min^{-1})*	*Dominant blood flow (ml 100 ml^{-1}min^{-1})*	*Plasma L-754,030 concentration‡ (ng ml^{-1})*	*MAP (mmHg)*	*HR (beats min^{-1})*	*Non-dominant arm blood flow (ml 100 ml^{-1}min^{-1})*	*Dominant arm blood flow (ml 100 ml^{-1}min^{-1})*	*Plasma L-754,030 concentration‡ (ng ml^{-1})*
First substance P challenge										
Placebo	90 ± 2	55 ± 3	2.9 ± 0.2	2.9 ± 0.4	–	88 ± 2	60 ± 4	12.3 ± 1.5*	2.9 ± 0.3	–
5 mg	85 ± 2	57 ± 2	3.5 ± 0.4	3.0 ± 0.5	–	89 ± 2	59 ± 2	16.6 ± 2.9*	3.0 ± 0.5	–
1 mg	87 ± 3	51 ± 1	3.2 ± 0.4	2.8 ± 0.4	–	91 ± 3	54 ± 2	9.7 ± 2.2*	2.8 ± 0.4	–
0.25 mg	90 ± 3	60 ± 2	3.0 ± 0.3	2.7 ± 0.3	–	91 ± 3	62 ± 4	12.1 ± 1.4*	2.7 ± 0.3	–
Second substance P challenge										
Placebo	95 ± 2	57 ± 4	3.4 ± 0.3	3.1 ± 0.3	–	93 ± 3	59 ± 3	20.1 ± 3.1*	5.8 ± 0.8†	
5 mg	88 ± 2	57 ± 3	3.6 ± 0.5	3.2 ± 0.4	75.4 ± 0.4	90 ± 2	57 ± 3	5.1 ± 0.6†	3.5 ± 0.4	86.1 ± 10.7
1 mg	90 ± 3	54 ± 2	3.3 ± 0.4	3.0 ± 0.3	10.0 ± 1.1	92 ± 3	58 ± 3	6.0 ± 0.9*	3.0 ± 0.4	11.1 ± 1.0
0.25 mg	92 ± 2	56 ± 4	2.7 ± 0.2	2.8 ± 0.3	2.6 ± 0.2	93 ± 3	64 ± 4	7.8 ± 1.2*	2.8 ± 0.3	2.8 ± 0.2
Part 2										
Placebo	86 ± 2	55 ± 3	3.3 ± 0.4	2.7 ± 0.4	–	88 ± 2	61 ± 5	22.7 ± 2.0*	7.3 ± 0.8*	–
1.43 mg	87 ± 4	55 ± 2	3.2 ± 0.4	2.6 ± 0.3	2.3 ± 0.3	86 ± 3	57 ± 2	11.9 ± 1.8*	3.5 ± 0.53†	2.2 ± 0.3

*$P < 0.001$ (*vs* baseline; *t*-test); †$P < 0.05$ (*vs* baseline; *t*-test). ‡$n = 8$ for 0.25 mg, $n = 7$ for 1 mg, $n = 8$ for 1.43 mg and $n = 2$ for 5 mg of L-758 298. §Maximum substance P dose: 8 pmol min^{-1} for part 1, pre-infusion. 128 pmol min^{-1} for part 1, during infusion. 128 pmol min^{-1} for part 2.

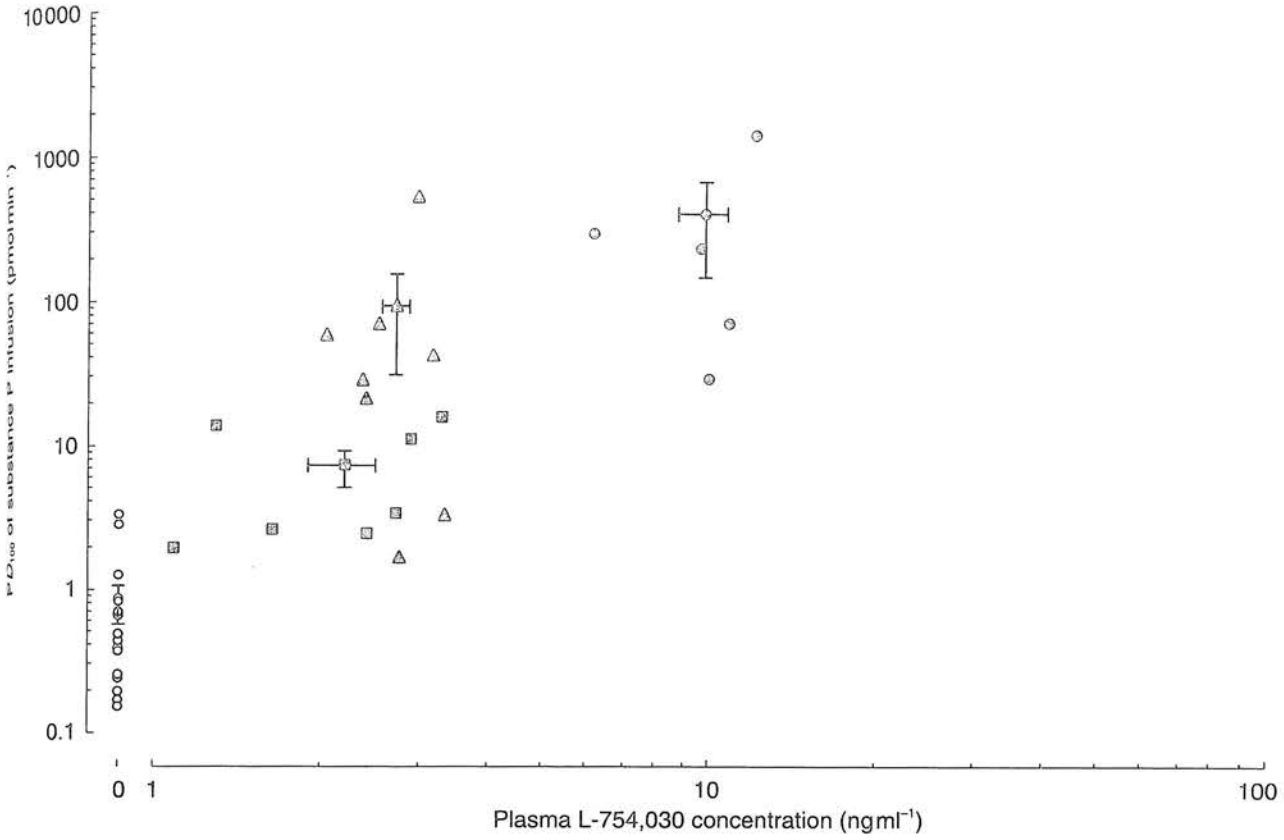

Figure 4 Concentration–response relationship between the plasma concentration of L-754 030 and the PD_{100} values for the rate of substance P infusion. Individual values shown with mean ± s.e.mean. Three points with indeterminate PD_{100} values >1454 are not shown. Placebo; ○ 0.25 mg, △ 1 mg and ▨ 24 h after 1.43 mg of L-758 298 $r=0.62$, $P=0.003$.

substance P induced vasodilatation was significantly inhibited (Table 2; Figure 3). The geometric mean PD_{100} (95% CI) was increased by 34–fold (4–299; $P<0.008$). The PD_{100} and plasma L-754 030 concentrations (Table 2) were similar to those obtained with 0.25 mg of L-758 298 in part 1.

Forearm blood flow in the dominant arm increased at doses ≥ 32 pmol min^{-1} following placebo ($P<0.02$ *vs* baseline; paired *t*-test) and at 128 pmol min^{-1} following 1.43 mg of L-758 298 ($P=0.03$ *vs* baseline; paired *t*-test). Heart rate and blood pressure did not change significantly (Table 2).

Concentration-response relationship

The logarithm of the mean plasma L-754 030 concentrations significantly correlated with the logarithm of the PD_{100} of the rate substance P infusion ($r=0.62$, $P=0.003$; Figure 4).

Tolerability

L-758 298 was generally well tolerated by all the subjects, with no excess of adverse events (mild headaches and back pain) in comparison with placebo. There were no serious adverse events during the study and no clinically significant abnormalities detected on safety monitoring (urinalysis, haematology, clinical chemistry and electrocardiography).

Discussion

For the first time, we have shown that substance P induced forearm vasodilatation is inhibited by a selective NK_1 receptor antagonist *in vivo* in man. During L-758 298 infusion, substance P induced forearm vasodilatation was inhibited in a dose dependent manner. At the highest dose of L-758 298, the vasodilator response to substance P was abolished at doses up to 8 pmol min^{-1}, suggesting that substance P mediated vasodilatation is entirely dependent on the endothelial NK_1 receptor. Moreover, persistent inhibition of substance P induced vasodilatation was present 24 h after L-758 298 infusion.

Despite previous proposals [17, 18], it would appear that substance P, acting via the NK_1 receptor, does not play a role in the regulation of peripheral vascular tone or blood pressure. We observed no alterations in baseline forearm blood flow or systemic haemodynamics following

L-758 298 infusion despite a greater than 1454-fold shift in the PD_{100} for substance P induced forearm vasodilatation. The 95% confidence intervals indicate that if substance P provides any contribution to basal peripheral vascular tone or systemic haemodynamics then it is rather small.

We have previously shown that repeated responses to substance P are reproducible and well tolerated [24]. In the current study, we have again seen good reproducibility of both within-day and between-day responses to intra-arterial substance P infusions. Systemic effects, such as increases in contralateral forearm blood flow, were also observed at substance P doses of ≥ 32 pmol min^{-1} without significant changes in heart rate or blood pressure. Moreover, these increases in contralateral forearm blood flow were also inhibited by L-758 298 infusion. Finally, L-758 298 infusion was generally well tolerated without any significant adverse events.

Study limitations

Because L-758 298 was administered systemically, there remains the possibility that compensatory mechanisms may have obscured a potential haemodynamic effect. Direct intra-arterial administration of an NK_1 receptor antagonist would provide a more precise method of assessing the role of substance P in the regulation of vascular tone. However, L-758 298 is a prodrug which requires conversion by systemic hepatic phosphatases to the active form L-754 030 and its intra-arterial administration would therefore not result in local NK_1 receptor antagonism.

In the present study, we did not assess the selectivity of L-754 030 for the NK_1 receptor by comparing substance P induced vasodilatation with an alternative non-NK_1 receptor mediated, endothelium-dependent vasodilator, such as bradykinin or acetylcholine, and this requires confirmation in future studies. However, L-754 030 has been shown to be highly selective for the NK_1 receptor [20].

We conclude that substance P induced forearm vasodilatation is mediated by the endothelial cell NK_1 receptor in man. Endogenous substance P does not appear to contribute to the maintenance of peripheral vascular tone or systemic blood pressure. In this study, intravenous L-758 298 was generally well tolerated with L-754 030 causing long lasting and potent NK_1 receptor antagonism in man.

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An *in vivo* Model for the Assessment of Acute Fibrinolytic Capacity of the Endothelium

David E. Newby[1, 2], Robert A. Wright[2], Christopher A. Ludlam[3], Keith A. A. Fox[2], Nicholas A. Boon[2], David J. Webb[1]

From the [1]Clinical Pharmacology Unit and Research Centre, University of Edinburgh, Western General Hospital, Edinburgh, UK, [2]Department of Cardiology, University of Edinburgh, Royal Infirmary, Edinburgh, UK, [3]Department of Haematology, University of Edinburgh, Royal Infirmary, Edinburgh, UK

Summary

The effects on blood flow and plasma fibrinolytic and coagulation parameters of intraarterial substance P, an endothelium dependent vasodilator, and sodium nitroprusside, a control endothelium independent vasodilator, were studied in the human forearm circulation. At subsystemic locally active doses, both substance P (2-8 pmol/min) and sodium nitroprusside (2-8 μg/min) caused dose-dependent vasodilatation ($p < 0.001$ for both) without affecting plasma concentrations of PAI-1, von Willebrand factor antigen or factor VIII:C activity. Substance P caused local increases in t-PA antigen and activity ($p < 0.001$) in the infused arm while sodium nitroprusside did not. At higher doses, substance P increased blood flow and t-PA concentrations in the noninfused arm. We conclude that brief, locally active and subsystemic infusions of intraarterial substance P cause a rapid and substantial local release of t-PA which appear to act via a flow and nitric oxide independent mechanism. This model should provide a useful and selective method of assessing the *in vivo* capacity of the forearm endothelium to release t-PA acutely.

Introduction

Endothelial cells of the precapillary arterioles and postcapillary venules (1) synthesise and constitutively secrete tissue-type plasminogen activator (t-PA) and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1). The release of t-PA may be rapidly increased through the translocation of a dynamic intracellular storage pool (2) in response to stimulation by blood coagulation and humoral factors (3). Acute t-PA release plays a pivotal role in endogenous fibrinolysis and this is exemplified by a plasminogen activator deficient gene knockout mouse model that exhibits an increased incidence of endotoxin-induced thrombosis (4). The time course of t-PA release is important, with thrombus dissolution being much more effective if t-PA is incorporated during, rather than after, thrombus formation (5, 6). Thus, the speed with which, and extent to which, t-PA can be released from endothelial cells may have a substantial impact on the efficacy of endogenous fibrinolysis.

When studying *in vivo* vascular responses in man, systemic drug administration can cause concomitant effects on other organ systems, such as the liver, brain, kidney and heart, as well as influence neurohumoral reflexes through changes in systemic haemodynamics. Therefore, because of these confounding influences, vascular and humoral responses cannot be wholly attributed to a direct effect of the drug on the blood vessels. Endogenous fibrinolysis in man has been assessed using systemic infusion of agents such as desmopressin (7, 8) and angiotensin II (9). These agents are vasoactive, producing changes in blood pressure and regional blood flow, as well as having widespread effects on many tissues. Thus, changes in systemic fibrinolytic parameters might be attributable to a number of factors including changes in hepatic release and clearance of t-PA and PAI-1, and the concomitant release of other stimulatory, vasoactive and humoral mediators. In contrast, the use of bilateral forearm blood flow measurements coupled with unilateral brachial artery infusion of vasoactive drugs at subsystemic, locally active doses, provides a powerful and reproducible method of directly assessing vascular responses *in vivo* (10, 11). Combined with bilateral forearm venous sampling, this technique permits the assessment of local release of tissue and endothelium-derived factors (12).

Substance P is a member of the tachykinin family of peptides, acting through stimulation of neurokinin receptors, and having a particularly high affinity for the type 1 (NK_1) receptor (13). It is widely distributed in the body and has actions as a central, peripheral and enteric neurotransmitter (14-17), inflammatory mediator (18-20) and neurohumoral regulator (15, 21, 22). When given intra-arterially in man, substance P enhances local fibrinolytic activity through an unknown mechanism (23) and has actions as a potent vasodilator (24-26) through an endothelium dependent (27) and predominantly nitric oxide mediated mechanism (28, 29). The determination of vascular responses to the intraarterial infusion of endothelial cell stimulants such as substance P has been widely used to assess the integrity of endothelium dependent vasodilatation in health and disease (30-33).

The initial aim of the present study was to assess the acute release of coagulation and fibrinolytic factors within the forearm vascular bed in response to intraarterial substance P using an ascending dose design to define the dose at which systemic effects intervene. Thereafter, endothelial cell release of these factors was assessed in response to locally active doses of substance P and a control endothelium-independent nitric oxide donor, sodium nitroprusside (32, 33).

Materials and Methods

Subjects

Sixteen healthy non-smoking men aged between 20 and 34 years participated in two studies which were 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. None of the subjects received vasoactive or nonsteroidal antiinflammatory drugs in the week before each phase of the study, and all abstained from

Correspondence to: Dr. D. E. Newby, Clinical Pharmacology Unit and Research Centre, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, Scotland, UK – Tel. +44 131 332 1205; FAX Number +44 131 343 6017; E-mail D.E.Newby@ed.ac.uk

alcohol for 24 h, and from food and caffeine-containing drinks for at least 5 h, before each study. All studies were performed in a quiet, temperature controlled room maintained at 23.5-24.5° C.

Intraarterial Administration

The brachial artery of the nondominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under 1% lignocaine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, UK) local anaesthesia and attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, UK). Patency was maintained by infusion of saline via an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 ml/min.

Drugs

Pharmaceutical-grade substance P (Clinalfa AG, Läufelfingen, Switzerland) and sodium nitroprusside (Nipride; Roche, Welwyn Garden City, UK) were administered following dissolution in saline (0.9%: Baxter Healthcare Ltd, Thetford, UK).

Forearm Blood Flow and Blood Pressure

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges applied to the widest part of the forearm (10, 11). 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 for 10 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 Strain Gauge Plethysmograph (D.E. Hokanson) was processed by a MacLab® analogue-to-digital converter and Chart v3.3.8 software (AD Instruments Ltd, Castle Hill, Australia) and recorded onto a MacIntosh Classic II computer (Apple Computers Inc, Cupertino, USA). Calibration was achieved using the internal standard of the plethysmograph.

Blood pressure was monitored in the noninfused arm at intervals throughout each study using a semiautomated noninvasive oscillometric sphygmomanometer (34) (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan).

Venous Sampling and Assays

Venous cannulae (19G) were inserted into large subcutaneous veins of the antecubital fossa in both arms as described previously (12). Ten ml of blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool® Stabilyte™, Umeå, Sweden) and citrate (Monovette®, Sarstedt, Nümbrecht, Germany) tubes, and kept on ice before being centrifuged at 2,000 g for 30 min at 4° C. Platelet free plasma was decanted and stored at –80° C before assay.

Plasma PAI-1 and t-PA antigen concentrations were determined using an enzyme-linked immunosorbent assay; Coaliza® PAI-1 (35) and Coaliza® t-PA (36) (Chromogenix AB, Mölndal, Sweden) respectively. Plasma PAI-1 and t-PA activities were determined by a photometric method, Coatest® PAI-1 (37) and Coaset® t-PA (38) (Chromogenix AB). Intraassay 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. Interassay coefficients of variability were 4.0%, 7.3%, 4.0% and 7.6% respectively. The sensitivities of the assays were 2.5 ng/ml, 0.5 ng/ml, 5 AU/ml and 0.10 IU/ml respectively. Von Willebrand factor (vWf) antigen was determined (39) using an enzyme-linked immunosorbent assay (Dako A/S, Glostrup, Denmark) with a sensitivity of 0.05 IU/ml. The intra-assay and interassay coefficients of variability were 5.2% and 7.3% respectively. Factor VIII:C procoagulant activity was determined using a standard one stage assay on an ACL-3000+ coagulometer (Instrumentation Laboratory, Warrington, UK).

Study Design

Subjects rested recumbent throughout each study. Strain gauges and cuffs were applied and the brachial artery of the nondominant arm cannulated. Measurements of forearm blood flow were made between 3 and 6 min of each infusion period unless otherwise stated. Before participating in one of the following protocols, saline was infused for the first 30 min to allow time for

Table 1 Dose ranging study: systemic haemodynamics, forearm blood flow, estimated net t-PA antigen and activity release, and vWf concentrations and factor VIII:C activity in the infused and non-infused forearms at baseline and during substance P infusion (n = 7). * p = 0.05 (t-test); ‡ p <0.001 (ANOVA); § p = 0.06 (ANOVA)

		Substance P Infusion (pmol/min)			
		Baseline	**2**	**16**	**64**
Blood Pressure (mmHg)	**Systolic**	140 ± 6	139 ± 4	139 ± 4	138 ± 7
	Diastolic	70 ± 4	68 ± 5	69 ± 6	65 ± 4
Heart Rate (/min)		57 ± 4	56 ± 4	61 ± 3	63 ± 3
Percentage Change in Forearm Blood Flow		-	233 ± 59 %	466 ± 197 %	221 ± 105 %‡
Absolute Forearm Blood Flow (ml/100 ml/min)	**Non-infused Arm**	3.4 ± 0.4	3.6 ± 0.5	4.6 ± 0.9*	8.8 ± 1.7‡
	Infused Arm	3.7 ± 0.7	11.7 ± 1.5	20.9 ± 2.8	21.5 ± 2.4‡
Estimated Net Release	**t-PA Antigen** (ng/100ml/min)	0.7 ± 0.8	3.8 ± 3.1	18.2 ± 5.3	78.4 ± 25.3‡
	t-PA Activity (IU/100ml/min)	-0.1 ± 0.1	0.9 ± 0.6	20.0 ± 7.8	45.6 ±9.6‡
von Willebrand Factor (IU/ml)	**Non-infused Arm**	0.72 ± 0.06	0.84 ± 0.07	0.99 ± 0.04	0.89 ± 0.04§
	Infused Arm	0.81 ± 0.07	0.93 ± 0.09	1.04 ± 0.15	1.02 ± 0.15
Factor VIII:C (IU/ml)	**Non-infused Arm**	0.49 ± 0.05	0.56 ± 0.05	0.58 ± 0.05	0.53 ± 0.05
	Infused Arm	0.52 ± 0.03	0.57 ± 0.04	0.56 ± 0.05	0.49 ± 0.03

equilibration, with forearm blood flow measured every 10 min and the final measurement taken as basal blood flow.

Dose Ranging Study

In seven men, intrabrachial substance P was administered in incremental doubling doses from 0.5 to a maximum of 128 pmol/min for 6 min at each dose and was followed by 30 min saline infusion. Venous samples were taken at baseline, following 2 pmol/min, 16 pmol/min and the maximal dose of substance P, and after the final 30 min saline infusion.

Local Forearm Study

Twelve men were given intraarterial doubling doses of substance P at 2, 4 and 8 pmol/min for 10 min at each dose, and sodium nitroprusside at 2, 4 and 8 μg/min for 10 min at each dose, separated by a 30 min saline infusion. Substance P and sodium nitroprusside were given single blind, in randomised order. Venous samples were obtained at the end of each period of saline infusion and with each dose of substance P and sodium nitroprusside.

Data Analysis and Statistics

Plethysmographic data were extracted from the Chart data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel v4.0; Microsoft Corporation, Cambridge, USA). Recordings from the first 60 s after wrist cuff inflation were not used because of the reflex vasoconstriction this causes (10, 11). Usually, the last five flow recordings in each 3 min measurement period were calculated and averaged for each arm. To reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point; in effect using the non-infused arm as a contemporaneous control for the infused arm (10, 11).

Estimated net release of t-PA activity and antigen was defined as the product of the infused forearm plasma flow (based on the hematocrit, HCt and the infused forearm blood flow, FBF) and the concentration difference between the infused ($[t\text{-}PA]_{Inf}$) and non-infused arms ($[t\text{-}PA]_{Noninf}$).

$$\text{Estimated net t-PA release} = FBF \times \{1\text{-}HCt\} \times \{[t\text{-}PA]_{Inf} - [t\text{-}PA]_{Noninf}\}$$

Data were examined, where appropriate, by two way analysis of variance (ANOVA) with repeated measures and two tailed paired Student's *t*-test using Excel v4.0 (Microsoft). All results are expressed as means ± standard errors of the mean. Statistical significance was taken at the 5% level.

Results

All subjects were normotensive (Tables 1 and 2) and had a normal fasting lipid profile with a mean total cholesterol concentration of 3.90 ± 0.16 mM (150 ± 6 mg/dl), high density lipoprotein cholesterol concentration of 1.02 ± 0.05 mM (39 ± 2 mg/dl) and triglyceride concentration of 0.87 ± 0.08 mM (77 ± 7 mg/dl).

Dose Ranging Study

There were no significant changes in arterial pressure or heart rate throughout the study. Substance P caused an increase in blood flow of the infused forearm ($p < 0.001$) from a baseline of 3.7 ± 0.7 ml/100ml/min to a maximum of 22 ± 2.4 ml/100ml/min at 64 pmol/min in a dose-dependent manner (Fig. 1). Five subjects received 64 pmol/min and 2 received 128 pmol/min as the maximum dose, further infusion of substance P being discontinued because of forearm skin oedema and facial flushing. There was a significant

Table 2 Local forearm study: systemic haemodynamics, forearm blood flow, estimated net t-PA antigen and activity release, and vWf concentrations and factor VIII : C activity in the infused and non-infused forearms at baseline and during sodium nitroprusside and substance P infusion (n = 12). * $p < 0.001$ (ANOVA)

		Sodium Nitroprusside (μg/min)					Substance P (pmol/min)			
		0	2	4	8		0	2	4	8
Blood Pressure (mmHg)	Systolic	132 ± 4	133 ± 4	135 ± 4	133 ± 4		131 ± 3	138 ± 4	135 ± 3	136 ± 3
	Diastolic	70 ± 2	70 ± 2	69 ± 2	68 ± 1		70 ± 2	71 ± 3	70 ± 2	68 ± 3
Heart Rate (/min)		63 ± 2	61 ± 2	61 ± 2	61 ± 2		64 ± 2	61 ± 2	61 ± 2	60 ± 2
Percentage Change in Forearm Blood Flow		-	247 ± 53	370 ± 73	541 ± 111	*	-	193 ± 41	286 ± 77	383 ± 79
Absolute Forearm Blood Flow (ml/100 ml/min)	Non-infused Arm	4.1 ± 0.5	4.1 ± 0.6	3.8 ± 0.6	3.8 ± 0.6		3.8 ± 0.6	4.0 ± 0.6	4.0 ± 0.6	3.9 ± 0.7
	Infused Arm	4.6 ± 0.7	11.6 ± 0.8	14.8 ± 1.1	18.1 ± 1.4	*	4.4 ± 0.6	11.1 ± 1.2	13.5 ± 1.4	16.5 ± 1.7
Estimated Net Release	t-PA Antigen (ng/100ml/min)	-0.5 ± 0.3	-1.6 ± 0.8	-0.5 ± 1.4	0.5 ± 2.7		0.0 ± 0.2	2.9 ± 1.3	7.3 ± 2.1	15.6 ± 3.7
	t-PA Activity (IU/100ml/min)	-0.2 ± 0.1	-0.8 ± 1.0	-0.7 ± 1.7	-1.1 ± 3.5		0.3 ± 0.2	5.0 ± 1.7	8.8 ± 2.5	17.8 ± 3.9
von Willebrand Factor (IU/ml)	Non-infused Arm	0.66 ± 0.08	0.60 ± 0.07	0.64 ± 0.10	0.68 ± 0.08		0.65 ± 0.10	0.59 ± 0.08	0.61 ± 0.10	0.60 ± 0.13
	Infused Arm	0.64 ± 0.08	0.62 ± 0.09	0.65 ± 0.09	0.67 ± 0.09		0.62 ± 0.07	0.58 ± 0.09	0.58 ± 0.10	0.63 ± 0.08
Factor VIII:C (IU/ml)	Non-infused Arm	0.61 ± 0.08	0.57 ± 0.08	0.59 ± 0.08	0.62 ± 0.08		0.57 ± 0.09	0.60 ± 0.08	0.59 ± 0.09	0.60 ± 0.09
	Infused Arm	0.64 ± 0.07	0.64 ± 0.09	0.65 ± 0.09	0.67 ± 0.09		0.53 ± 0.08	0.57 ± 0.10	0.57 ± 0.10	0.63 ± 0.09

ncrease in the blood flow of the contralateral, noninfused arm (p = 0.001, ANOVA) which was apparent from 16 pmol/min (p = 0.05). The relative percentage increase in blood flow of the infused compared with the noninfused arm was dose-dependent, peaking at 32 pmol/min before declining at 64 pmol/min (Fig. 1).

Substance P caused increases in plasma t-PA antigen and activity concentrations in the infused ($p < 0.001$ for both) and noninfused arm ($p \leq 0.003$ for both) which were dose-dependent (Fig. 2). Plasma from the infused arm demonstrated significantly greater increases in both t-PA activity and antigen concentrations than the noninfused arm ($p < 0.001$). At the maximal dose, mean t-PA activity increased by 930% in the infused arm and 210% in the noninfused arm, whilst mean t-PA antigen increased by 240% and 62% respectively.

There were no significant or consistent changes in plasma PAI-1 antigen or activity concentrations in the infused arm. There was a significant decrease in the plasma PAI-1 activity in the noninfused arm ($p = 0.03$) although PAI-1 antigen concentrations did not change significantly ($p = 0.64$). There were no significant changes in plasma vWf concentration or factor VIII:C activity in either arm (Table 1).

Local Forearm Study

There were no significant changes in blood pressure, heart rate or forearm blood flow in the contralateral arm throughout the study (Table 2).

Both substance P and sodium nitroprusside caused selective increases in forearm blood flow in the infused arm ($p < 0.001$ for both) in a dose dependent manner (Table 2). Substance P caused a selective and dose dependent increase in the estimated net release ($p < 0.001$ for both) and venous plasma concentrations ($p < 0.001$ for both) of both t-PA activity and antigen (Table 2; Fig. 3). In contrast, there were no significant changes in plasma t-PA activity or antigen concentrations in the noninfused arm, or in PAI-1 antigen and activity, vWf or factor VIII:C concentrations in either arm (Table 2; Fig. 3). There were no significant changes in t-PA, PAI-1, vWf or factor VIII:C in either arm during sodium nitroprusside infusion (Table 2; Fig. 3).

Discussion

We have shown, for the first time, that intraarterial substance P administration causes acute, selective and substantial t-PA release *in vivo* in man. At both systemic and locally active doses, substance P causes t-PA release from the forearm vascular bed without significant effects on the release of PAI-1, vWf and factor VIII:C. This model provides a selective *in vivo* method of assessing acute t-PA release from the endothelium in man.

Intrabrachial substance P has been shown previously to induce local fibrinolysis in the forearm (23) although the mechanism of this effect had not been determined. However, taken together with our findings, it is apparent that this enhancement of fibrinolytic activity is, at least in part, mediated through t-PA release. Previously, bradykinin was thought to be one of the most potent agents causing t-PA release in animals (3, 40) and man (3, 41). However, a recent study in man (41) using systemic intravenous bradykinin administration at doses of up to 380 pmol/kg/min, did not show a significant release of t-PA antigen except in the presence of angiotensin converting enzyme inhibition and alterations in systemic haemodynamic parameters. Jern and colleagues (42) have shown a significant net local release of t-PA antigen and activity in response to intra-brachial methacholine. However, a significant increase in venous concentrations of t-PA antigen was not detected and, although measurement of arteriovenous differences should enhance the accuracy of assessing local tissue release, there were no significant increases in the arteriovenous gradients of t-PA antigen or activity. Indeed, the clearest changes were observed in the arterial t-PA activity which should have remained constant, suggesting that there was systemic stimulation of t-PA release in this study.

Venous plasma t-PA concentrations obtained from a given tissue bed are composed of three components; circulating arterial t-PA, basal or constitutive endothelial cell release of t-PA and facultative or stimulated endothelial cell release of t-PA. The net tissue release of t-PA is equivalent to the product of the plasma flow through the tissue and the arteriovenous difference in plasma t-PA concentrations across it. In the absence of endothelial cell stimulation, but with an increase in blood flow across the tissue bed, venous plasma t-PA concentrations would be expected to fall secondary to a dilutional effect. However, this ignores the potential for clearance of t-PA across the vascular bed (43), and stimulation of its release by shear stress and flow (44, 45). Without measuring the arteriovenous concentration gradient across the forearm, net tissue release can only be derived and estimated. However, arterial sampling requires the insertion of large bore cannulae which do not lend themselves to multiple cannulations within the same subject. There is also the potential to introduce artefact from the presence of a larger thrombogenic surface given that activated factor Xa is the most

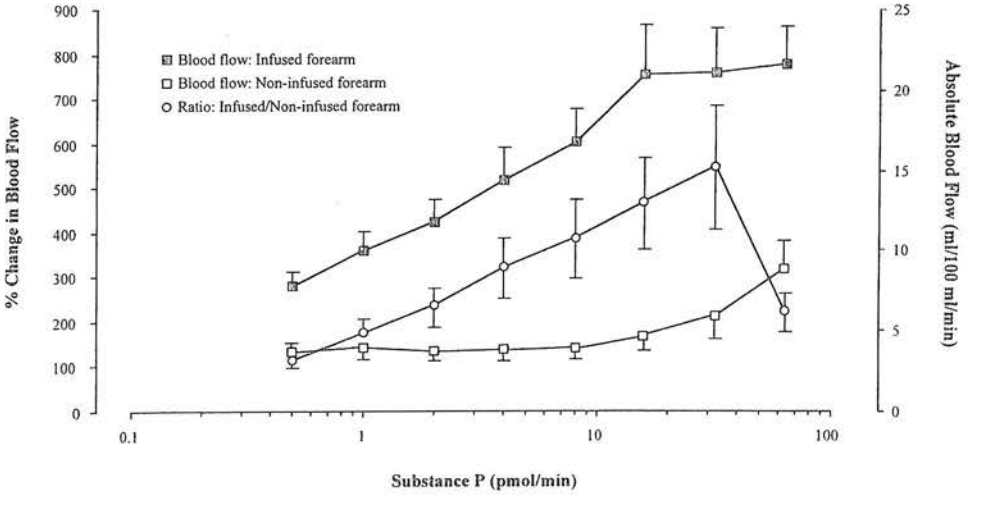

Fig. 1 Dose ranging study: percentage change and absolute forearm blood flow responses to incremental doses of substance P (n = 7).

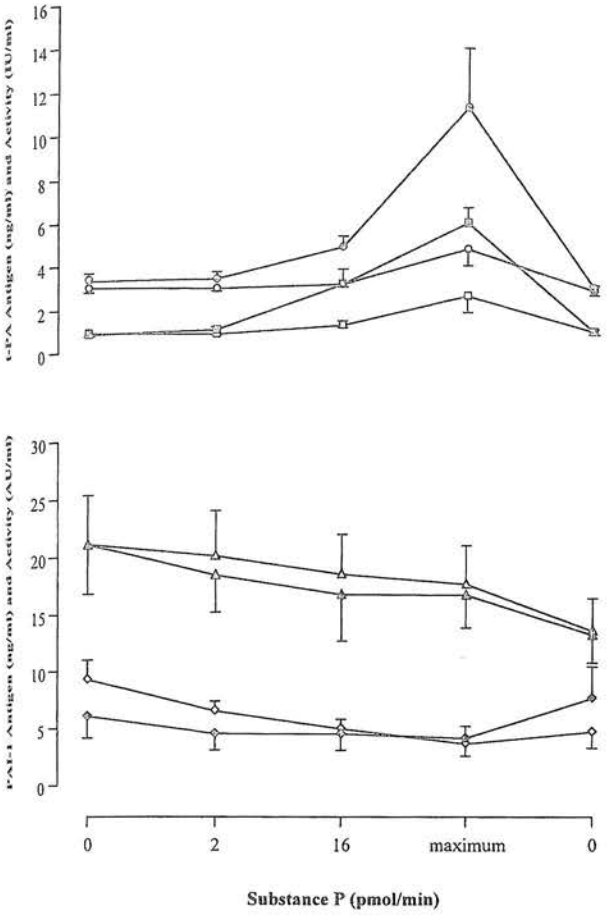

Fig. 2 Dose ranging study: venous plasma tissue plasminogen activator (t-PA) antigen (○) and activity (❑), and plasminogen activator inhibitor type 1 (PAI-1) antigen (Δ) and activity (✧) concentrations in the infused (closed symbols) and non-infused (open symbols) forearms in response to substance P infusions (n = 7). Maximum substance P dose was 64 pmol/min in 5 subjects and 128 pmol/min in 2 subjects. $p < 0.001$ for all t-PA concentrations (ANOVA)

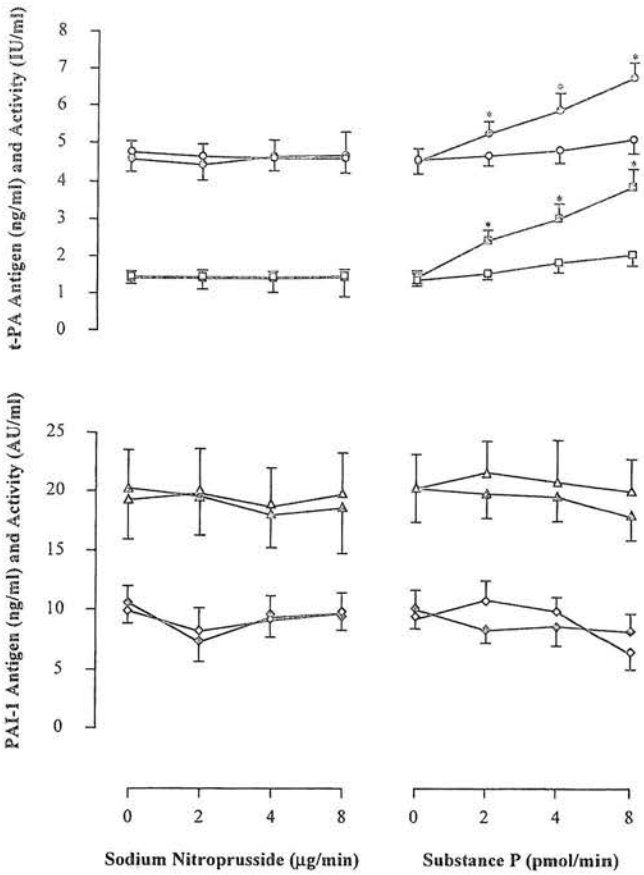

Fig. 3 Local forearm study: venous plasma tissue plasminogen activator (t-PA) antigen (○) and activity (❑), and plasminogen activator inhibitor type 1 (PAI-1) antigen (Δ) and activity (✧) concentrations in the infused (closed symbols) and non-infused (open symbols) forearms in response to sodium nitroprusside and substance P infusions (n = 12). * $p < 0.001$ (ANOVA)

potent stimulant for t-PA release yet known (3). Rather than assessing arteriovenous differences, we have compared venous plasma t-PA concentrations between infused and non-infused arms and have used very fine gauge arterial cannulae for drug administration only. This method may potentially underestimate the net release of t-PA and fail to detect a modest effect due to the potential flow dependent, dilutional changes in venous concentrations. However, typical resting arteriovenous differences are only ~10% of the total venous concentration (42, 46) and the basal constitutive release of t-PA antigen is 0.9 ng/100 ml of tissue/min in the forearm (42). Thus, in the presence of large increases in t-PA release, the dilutional effect of increased blood flow on constitutive t-PA release will be reduced. Indeed, using this bilateral venous sampling methodology, we have been able to demonstrate a substantial, dose-dependent release of t-PA from the forearm vascular bed in response to substance P infusion. Moreover, despite *in vitro* evidence that t-PA release may be influenced by shear stress (44, 45), we have found that the endothelium independent nitric oxide donor, sodium nitroprusside, has no significant effect on the venous t-PA concentrations despite comparable increases in blood flow to those with substance P. Sodium nitroprusside is known to have no direct effect on the endothelial cell release of t-PA and PAI-1 *in vitro* (47) and, therefore, it is likely that either shear stress and flow dependent stimulation of endothelial cell t-PA release is counterbalanced by the potential dilutional effects of increased flow, or that this theoretical flow dependence of venous concentrations is negligible. This also indicates that increases in nitric oxide and blood flow are not sufficient in themselves to release t-PA from the endothelium. However, it remains a possibility that the L-arginine : nitric oxide pathway plays a role in substance P-induced t-PA release and requires further studies using a combined infusion of substance P and a nitric oxide synthase inhibitor such as L-N^G-monomethyl arginine.

Although we have produced substantial increases in both t-PA activity and antigen, we did not detect release of PAI-1, or the coagulation factors, vWf and factor VIII : C. This would indicate that these agents are not stored in a rapidly translocatable pool within the endothelial cells of the forearm vascular bed or that they are not released in response to substance P over the time course and at the doses used here. However, protracted endothelial cell stimulation may release these factors (7, 9). In this respect, it is interesting to note the time dependent reduction in PAI-1 concentrations seen in our studies, although these only achieved statistical significance in the non-infused arm during the dose ranging study. The most likely explanation for a reduction in PAI-1 is that the released and active t-PA is complexed by circulating PAI-1 and subsequently cleared from the circulation by the liver (48).

Thus, substantial local t-PA release will tend to reduce systemic PAI-1 concentrations in the short term, as seen with the administration of pharmacological doses of t-PA (49).

As anticipated, local substance P infusion did not affect the rate of release of hepatically derived factor VIII : C. In contrast, it is perhaps surprising that we did not observe a rise in plasma vWf concentrations to accompany the release of t-PA. To date, stimulation of t-PA release using a wide range of secretagogues such as thrombin, vasopressin, bradykinin, histamine and desmopressin, has invariably been accompanied by concomitant vWf release (7, 50, 51). However, we were unable to detect an acute local release of vWf even in the presence of high local concentrations of substance P in the dose ranging study. This novel selectivity suggests that the endothelium is able to mobilise different cytoplasmic storage pools in response to specific (NK_1) receptor stimulation. Further studies with more prolonged infusions of substance P would be required to determine whether the endothelial cell vWf or PAI-1 release is delayed or is truly not influenced by substance P.

In summary, brief, locally active and subsystemic infusions of intra-arterial substance P produce a rapid and substantial increase in plasma t-PA activity and antigen concentrations across the forearm bed which appear to act via a flow and nitric oxide independent mechanism. This model provides a powerful method of assessing the *in vivo* capacity of the endothelium to acutely release t-PA within the forearm vascular bed and would be applicable to the assessment of diseases associated with endothelial dysfunction, such as hypercholesterolemia (52).

Acknowledgements

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Endothelin-1 does not contribute to the release of tissue plasminogen activator in vivo in man

D.E Newby, F.E. Strachan, N.R. Johnston, D.J. Webb

Clinical Pharmacology Unit and Research Centre, University of Edinburgh, Western General Hospital, Edinburgh, UK

Summary *Objectives*: Endothelin-1 is a potent endothelium-derived vasoconstrictor peptide with autocrine and paracrine actions. Tissue plasminogen activator (t-PA) and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1), are also released from the vascular endothelium and play a pivotal role in endogenous fibrinolysis. We, therefore, examined the effects of exogenous and endogenous endothelin-1 on t-PA and PAI-1 release in vivo in man.
Design: Open investigative study.
Setting: Clinical Research Centre, University of Edinburgh.
Subjects: Fourteen healthy male volunteers.
Interventions: Unilateral brachial artery infusions of endothelin-1 at 2.5 and 10 pmol/min, and the selective endothelin type B (ET_B) receptor antagonist, BQ-788, at 1 nmol/min.
Main outcome measures: Blood flow and plasma fibrinolytic factors were measured in both forearms using venous occlusion plethysmography and venous blood samples withdrawn from the antecubital fossae.
Results: Endothelin-1 caused a slow onset dose-dependent forearm vasoconstriction ($P<0.001$) with a maximal reduction in blood flow of 40 ± 4% and 63 ± 3% at 2.5 and 10 pmol/min respectively. BQ-788 also caused a slow onset reduction in forearm blood flow ($P<0.001$) reaching a maximum of 21 ± 3%. However, BQ-788 and endothelin-1 did not affect plasma concentrations of t-PA or PAI-1 in the venous effluent of the infused forearm.
Conclusions: Despite sustaining significant vasoconstriction, neither endogenous nor exogenous endothelin-1 influences the release of t-PA or PAI-1 in the forearm vascular bed of man. This suggests that endothelin-1 does not provide a major contribution to the regulation of endogenous fibrinolysis in man. © Harcourt Publishers Ltd 1999

INTRODUCTION

Endothelial cells in the precapillary arterioles and post-capillary venules[1] synthesize and release t-PA and PAI-1 both basally and in response to stimulation by various coagulation factors and stimulants. The time course of t-PA release is important since clot dissolution is much more effective if t-PA is incorporated during clot formation rather than following completion.[2,3] The acute release of t-PA results from the rapid translocation of a dynamic intracellular storage pool[4] and plays a pivotal role in endogenous fibrinolysis.

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Correspondence to: Dr D.E. Newby, Cardiovascular Research Unit, Department of Cardiology, Royal Infirmary, Lauriston Place, Edinburgh, EH3 9YW, Scotland, UK. Tel: +44 (0) 131 536 2749; Fax: +44 (0) 131 536 2744; E-mail: D.E.Newby@ed.ac.uk

Endothelin-1 is a potent endothelium-derived vasoconstrictor peptide with autocrine and paracrine actions. It is continuously released by the endothelium and contributes to the maintenance of basal vascular tone[5] and blood pressure.[6] There are two main endothelin receptor subtypes, ET_A and ET_B, but only the ET_B receptors are present on the endothelium. Endothelin-1 causes vasoconstriction mainly through stimulation of the smooth muscle cell ET_A receptor, although smooth muscle ET_B receptors may also contribute in some vessel types. This vasoconstrictor response is modulated by autocrine endothelial cell ET_B receptor-mediated generation of the endothelium-derived vasodilators, nitric oxide and prostacyclin.

The importance of endogenous t-PA release is exemplified by the high rate of spontaneous reperfusion in the infarct-related artery after acute myocardial infarction, occurring in around 30% of patients within the first 12 h.[7–10]

Following an acute myocardial infarction, plasma endothelin-1 concentrations are elevated and provide an important prognostic marker of survival at 1 year.[10] Furthermore, on the basis of in vitro studies, it has been suggested that endothelin-1 may contribute to the regulation of endogenous fibrinolysis and t-PA release.[11–13] However, the evidence is contradictory, with endothelin-1 being found to either inhibit[13] or stimulate[11,12] endothelial cell t-PA release. The role of endothelin-1 in the regulation of endogenous fibrinolysis in man is currently unknown.

We,[14,15] and others,[16,17] have shown, using bilateral forearm venous occlusion plethysmography and unilateral brachial artery infusions, that the forearm release of t-PA and PAI-1 can be determined in vivo in man. Therefore, the aim of the current study was, using synthetic endothelin-1 peptide and the selective ET_B receptor antagonist, BQ-788, to determine whether endothelin-1, of exogenous or endogenous origin, acts via the endothelial ET_B receptor to regulate the release of t-PA or PAI-1 in vivo in man.

MATERIALS AND METHODS

Subjects

Fourteen healthy men aged between 20 and 33 years participated in three studies which were 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. None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 h, and from food, tobacco and caffeine-containing drinks for at least 9 h, before each study. All studies were performed in a quiet, temperature-controlled room maintained at 23.5–24.5°C.

Intra-arterial administration and drugs

The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under 1% 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 P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was kept constant throughout all studies at 1 mL/min. Endothelin-1 (Clinalfa AG, Läufelfingen, Switzerland) and BQ-788 (American Peptide Company, Sunnyvale, USA) were administered following dissolution in saline.

Forearm blood flow and blood pressure

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges applied to the widest part of the forearm.[18,19] 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 for 10 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 Strain Gauge Plethysmograph (D.E. Hokanson) was processed by a MacLab® analogue-to-digital converter and Chart *v*3.3.8 software (AD Instruments Ltd, Castle Hill, Australia) and recorded onto a Macintosh Classic II computer (Apple Computers Inc, Cupertino, USA). Calibration was achieved using the internal standard of the plethysmograph.

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).[20]

Venous sampling and assays

Venous cannulae (17G) were inserted into large subcutaneous veins of the antecubital fossa in both arms. Ten mL of blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool® Stabilyte™, Umeå, Sweden; for t-PA assays) and citrate (Monovette®, Sarstedt, Nümbrecht, Germany; for PAI-1 assays) tubes, and kept on ice before being centrifuged at 2000 g for 30 min at 4°C. Platelet-free plasma was decanted and stored at –80°C before assay.[21]

Plasma PAI-1 and t-PA antigen concentrations were determined using an enzyme-linked immunosorbent assay (ELISA); Coaliza® PAI-1 [22] and Coaliza® t-PA[23] (Chromogenix AB, Mölndal, Sweden) respectively. Plasma t-PA activities were determined by a photometric method, Coaset® t-PA[24] (Chromogenix AB). Intra-assay coefficients of variation were 7 and 5.5% for t-PA and PAI-1 antigen, and 4% for t-PA activity respectively. Inter-assay coefficients of variability were 4, 7.3 and 4% respectively. The sensitivities of the assays were 2.5 ng/mL, 0.5 ng/mL and 0.10 IU/mL respectively. Haematocrit was determined by capillary tube centrifugation of blood anticoagulated by ethylene diamine tetraacetic acid and was obtained from the infused forearm at baseline and at 120 min.

Study design

On each study day, subjects attended fasted and rested recumbent throughout the study. Strain gauges and cuffs

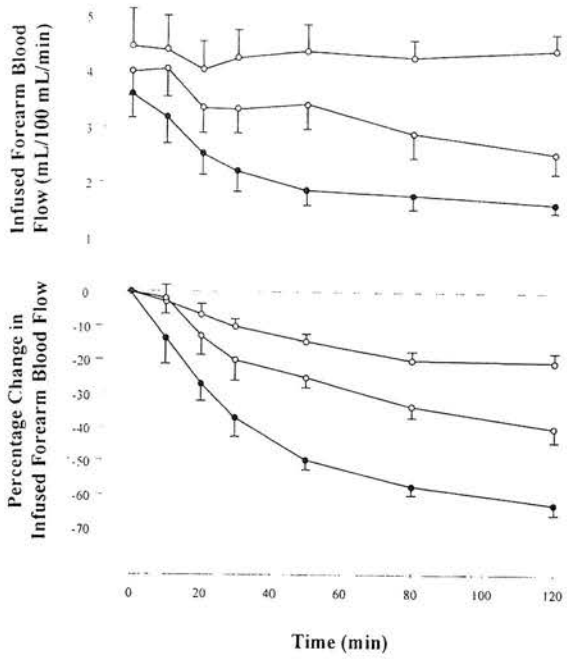

Fig. 1 Absolute (mL/100 mL of tissue/min: upper panel) and percentage (% relative to the non-infused forearm: lower panel) change of blood flow in the infused forearm during intra-arterial infusion of BQ-788 (1 nmol/min;○) and endothelin-1 (2.5 pmol/min:○ and 10 pmol/min: ●).

were applied and the brachial artery of the non-dominant arm cannulated. Throughout each of the studies, measurements of forearm blood flow were made every 10 min. Before drug administration, saline was infused for 30 min to allow time for equilibration and the final blood flow measurement during saline infusion was taken as the basal forearm blood flow.

Eight subjects received an intra-brachial infusion of endothelin-1 at 2.5 and 10 pmol/min for 120 min, given in random order, on two separate occasions, at least 1 week apart. Eight subjects (two had also attended for endothelin-1 infusions) received an intra-brachial infusion of BQ-788 at 1 nmol/min for 120 min. Venous samples were withdrawn from each arm at baseline and at 10, 20, 30, 50, 80 and 120 min after the start of endothelin-1 or BQ-788 infusion.

Data analysis and statistics

Plethysmographic data were extracted from the Chart data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel *v5*; Microsoft Corporation, Cambridge, USA). Recordings from the first 60 s after wrist cuff inflation were not used because of the reflex vasoconstriction this causes.[18,19] Usually, the last five flow recordings in each 3 min measurement period were calculated and averaged for each arm. To reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point: in effect using the non-infused arm as a contemporaneous control for the infused arm.[18,19] Percentage changes in the infused forearm blood flow were calculated[18,19] as follows:

$$\% \text{ Change in blood flow} = 100 \times \{I_t/NI_t - I_b/NI_b\} / I_b/NI_b$$

Where I_b and NI_b are the infused and non-infused forearm blood flows at baseline (time 0) respectively, and I_t and NI_t are the infused and non-infused forearm blood flows at a given time point respectively.

Estimated net release of t-PA activity and antigen was defined previously[14,15] as the product of the infused forearm plasma flow (based on the mean haematocrit, HCt,

Table 1 Systemic haemodynamics, forearm blood flow and haematocrit at baseline and after intra-arterial infusion for 120 min.

	BQ-788 1 nmol/min		Endothelin-1 2.5 pmol/min		Endothelin-1 10 pmol/min	
	Basal	Final	Basal	Final	Basal	Final
Blood pressure (mmHg)						
systolic	130 ± 5	134 ± 6	136 ± 3	140 ± 4	133 ± 4	133 ± 4
diastolic	75 ± 4	77 ± 4	72 ± 3	71 ± 3	70 ± 3	73 ± 4
Heart rate (/min)	59 ± 3	60 ± 3	62 ± 4	58 ± 4	61 ± 5	62 ± 4
Absolute forearm blood flow (mL/100 mL/min)						
non-infused arm	3.1 ± 0.4	3.8 ± 0.2	3.4 ± 0.3	3.7 ± 0.5	3.1 ± 0.3	4.1 ± 0.3
infused arm	4.5 ± 0.7	4.2 ± 0.4*	4.0 ± 0.4	2.5 ± 0.3*	3.6 ± 0.4	1.6 ± 0.1*
Ratio of infused/non-infused	1.35 ± 0.15	1.14 ± 0.06†	1.13 ± 0.03	0.42 ± 0.03†	1.05 ± 0.07	0.40 ± 0.03†
Haematocrit	0.41 ± 0.01	0.41 ± 0.01	0.42 ± 0.02	0.40 ± 0.02‡	0.41 ± 0.01	0.40 ± 0.01‡

*$P \leq 0.001$ (two-way ANOVA: infused vs non-infused)
†$P < 0.001$ (one-way ANOVA)
‡$P < 0.05$ (paired t-test: basal vs final)

and the infused forearm blood flow, FBF) and the concentration difference between the infused ($[t\text{-}PA]_{inf}$) and non-infused arms ($[t\text{-}PA]_{Non\text{-}inf}$).

$$\text{Estimated net t-PA release} = FBF \times \{1\text{-}HCt\} \times \{[t\text{-}PA]_{Inf} - [t\text{-}PA]_{Non\text{-}inf}\}$$

Data were examined by two way analysis of variance (ANOVA) with repeated measures and two-tailed paired Student's t-test using Excel *v*5.0 (Microsoft) where appropriate. All results are expressed as mean ± SEM. Statistical significance was taken at the 5% level. Based on previous data,[14,15] the study had 90% power to detect a 20% change in plasma t-PA concentrations between treatment periods at the 5% level.

RESULTS

All subjects were normotensive and there were no significant changes in blood pressure, heart rate or blood flow in the contralateral arm throughout any of the studies (Table 1). Haematocrit decreased slightly in each endothelin study (Table 1). Between the 3 protocols there were no significant differences in the baseline values of blood pressure, heart rate, forearm blood flow, haematocrit or plasma concentrations of t-PA and PAI-1.

Endothelin-1 infusions

Endothelin-1 decreased blood flow in the infused arm ($P<0.001$) in a dose-dependent manner (Fig. 1) reaching a minimum of 2.5 ± 0.3 mL/100 mL/min at 2.5 pmol/min and 1.6 ± 0.1 mL/100 mL/min at 10 pmol/min, after 120 min. This corresponds to a relative reduction in forearm blood flow of 40 ± 4% and 63 ± 3% respectively. The plasma concentrations of t-PA and PAI-1 did not change in the infused arm (Fig. 2) during endothelin-1 infusion at either concentration (P=NS; one-way ANOVA). In comparison to the non-infused arm, there was a trend (P=0.06; two-way ANOVA) for the infused forearm plasma t-PA antigen concentration to be greater with 10 pmol/min of endothelin-1. However, there were no significant differences in plasma concentrations of PAI-1 antigen (Table 2) or t-PA activity between the forearms.

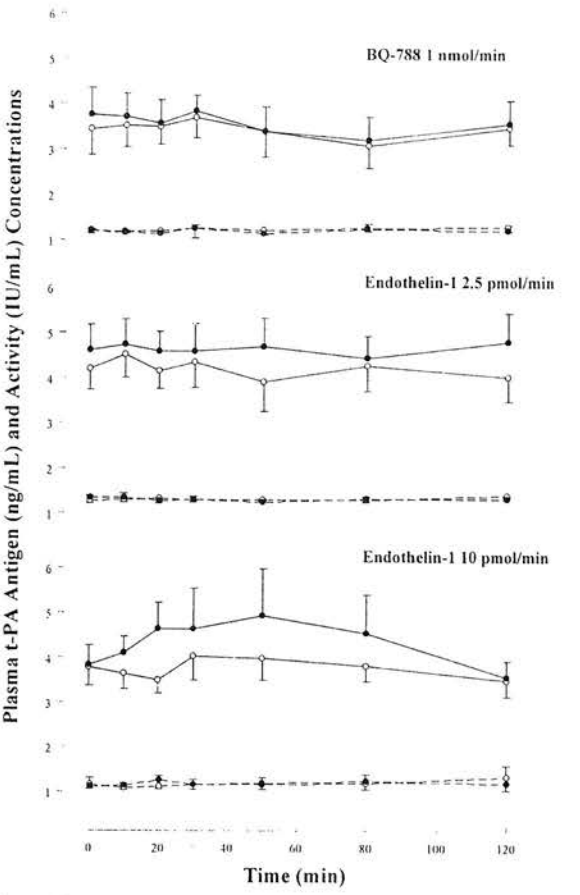

Fig. 2 Plasma concentrations of tissue plasminogen activator (t-PA) antigen (ng/mL; solid lines) and activity (IU/mL; dashed lines) in the infused (solid circles) and non-infused (open circles) forearm during intra-arterial infusion of BQ-788 (1 nmol/min) and endothelin-1 (2.5 and 10 pmol/min).

Table 2 Plasma plasminogen activator inhibitor type 1 (PAI-1) concentrations (ng/mL) during endothelin-1 (ET-1) and BQ-788 infusion. Mean ± SEM.

	Time (min)						
	Baseline	10	20	30	50	80	120
BQ-788 (1 nmol/min)							
infused arm	39 ± 15	37 ± 13	43 ± 15	41 ± 13	37 ± 12	37 ± 12	29 ± 9
non-infused arm	38 ± 13	40 ± 14	43 ± 14	41 ± 13	37 ± 13	33 ± 9	32 ± 9
ET-1 (2.5 pmol/min)							
infused arm	28 ± 7	27 ± 5	26 ± 6	26 ± 5	25 ± 5	21 ± 5	20 ± 5
non-infused arm	27 ± 5	29 ± 6	29 ± 6	27 ± 5	28 ± 7	25 ± 5	23 ± 4
ET-1 (10 pmol/min)							
infused arm	25 ± 5	26 ± 4	27 ± 5	26 ± 5	23 ± 5	21 ± 4	22 ± 5
non-infused arm	27 ± 5	27 ± 5	26 ± 5	25 ± 4	24 ± 5	23 ± 5	22 ± 4

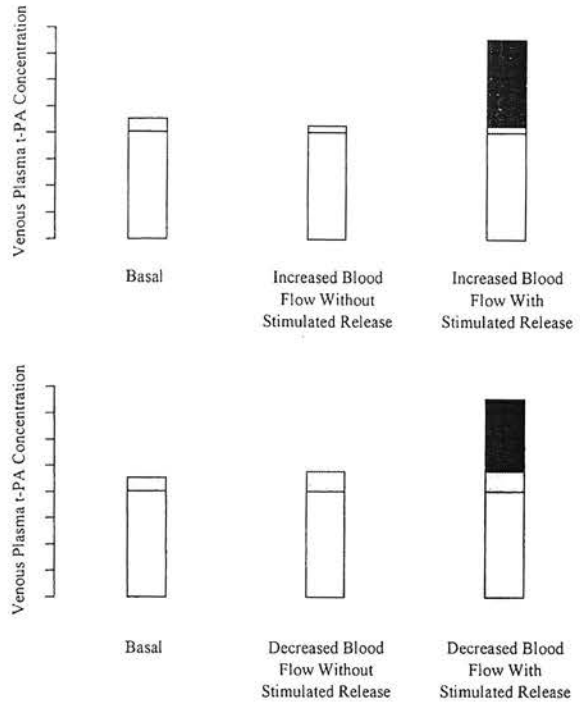

Fig. 3 Theoretical components of venous plasma t-PA concentration under basal conditions and during increases (upper panel) and decreases (lower panel) in blood flow with and without direct stimulation of t-PA release. Open bars: circulating or arterial t-PA; grey bars: basal 'constitutive' t-PA released from the tissue bed; black bars: stimulated 'facultative' t-PA released from the tissue bed

BQ-788 infusion

In comparison to the non-infused arm, BQ-788 decreased blood flow in the infused forearm after 120 min (relative reduction of 21 ± 3%) although the absolute blood flow was unchanged (Fig. 1 and Table 1). The plasma concentrations of t-PA and PAI-1 (Fig. 2 and Table 2) did not change in the infused forearm (P=NS; one-way ANOVA) or in comparison to the non-infused forearm (P=NS; two-way ANOVA).

There was no significant net release of t-PA with infusions of either endothelin-1 or BQ-788 (Table 3).

DISCUSSION

We have demonstrated that, despite causing significant reductions in blood flow, neither endogenous nor exogenous endothelin-1 influences the release of t-PA or PAI-1 in the forearm vascular bed of man. This suggests that endothelin-1 does not contribute to the regulation of endogenous fibrinolysis in man.

Endothelial cell culture techniques have limitations in the investigation of t-PA release and may not be truly representative of the in vivo function of these cells. The amount of t-PA released in culture is small and necessitates prolonged incubation periods and sensitive assays. Moreover, the phenotype of endothelial cells in culture, and the ability to release t-PA, changes with increasing passages. This may account for the disparity of our findings with previous endothelial cell culture studies.[13]

Studies in intact whole animals have suggested that systemic endothelin-1 infusion is associated with stimulation of t-PA release,[12] although plasma t-PA concentrations are not increased by low sub-pressor doses of endothelin-1 in man.[25] Systemic endothelin-1 administration, particularly at pressor doses, will induce changes in cardiac function and regional blood flow as well as having widespread effects on disparate tissues. Thus, the consequent changes in systemic fibrinolytic parameters will be a combination of many factors, potentially including hepatic production and clearance of t-PA and PAI-1. One approach, to avoid these confounding systemic effects, has been to use the isolated perfused rat hindlimb model. This ex vivo model has been reported to demonstrate that endothelin-1 infusion stimulates modest amounts of t-PA release.[11] However, this increased 'release' may, in part, reflect the concentrating effects of a reduction in blood flow associated with endothelin-1 infusion and the concentrations of endothelin-1 administered. In studies conducted to date,[11–13] endothelin-1 has been administered in nanomolar concentrations.

Table 3 Estimated net release of tissue plasminogen activator (t-PA) antigen across the forearm during endothelin-1 (ET-1) and BQ-788 infusion. Mean (95% confidence intervals)

	Time (min)						
	Baseline	**10**	**20**	**30**	**50**	**80**	**120**
t-PA release (ng/100 mL/min)							
BQ-788 (1 nmol/min)	1.1 (−0.1 to 2.3)	0 (−1.6 to 1.6)	0.4 (−1.0 to 1.8)	0.3 (−1.5 to 2.1)	−0.2 (−1.8 to 1.4)	0.1 (−1.3 to 1.5)	0.1 (−1.7 to 1.9)
ET-1 (2.5 pmol/min)	0.1 (−0.7 to 0.9)	1.4 (−0.6 to 3.4)	2.5 (0.5 to 4.5)	1 (−0.8 to 2.6)	2 (−1.0 to 5.0)	1.7 (−1.1 to 4.5)	0 (−0.2 to 0.2)
ET-1 (10 pmol/min)	1.3 (−0.3 to 2.9)	0.5 (−0.4 to 1.9)	0.9 (0.1 to 1.7)	0.3 (−0.4 to 1.7)	0.9 (−0.1 to 1.9)	0.1 (−0.7 to 0.9)	0.8 (0.0 to1.6)

Although local abluminal concentrations may be high, normal human plasma endothelin-1 concentrations are in the femtomolar range. Indeed, in the present study, assuming a total forearm blood flow of 30–50 mL/min, the forearm tissue concentration of endothelin-1 during the 10 pmol/min infusion will be 200–300 fmol/mL. The previous ex vivo animal studies,[11] therefore, represent some 4–5 orders of magnitude higher concentrations and the release of t-PA is likely to represent a pharmacological rather than physiological effect.

We have not detected a significant release of t-PA from the forearm with endothelin-1 infusion despite a 63% reduction in blood flow at the higher dose. Basal t-PA release is of the order of ~0.9 ng/100 mL of tissue/min in the forearm[16] and the apparent trend for an increase in t-PA antigen concentrations may, in part, reflect the reduction in blood flow associated with the marked forearm vasoconstriction (see Fig. 3). This is borne out by the unchanged t-PA activity, because it would be anticipated that plasma PAI-1 and t-PA antigen concentrations would increase proportionately with reductions in blood flow. ET_B receptor antagonism causes both inhibition of endothelium-derived vasodilators such as nitric oxide, and potential hyperstimulation of the unopposed ET_A receptor. However, as with endothelin-1, BQ-788 did not affect plasma concentrations of t-PA or PAI-1 in the infused forearm.

Forearm release of t-PA has been demonstrated using various endothelial cell stimulants including methacholine,[16,26] noradrenaline[17] and desmopressin.[27] Using the same technique as in the present study, we have previously demonstrated in vivo t-PA release of up to 80 ng/100 mL of tissue/min across the human forearm using intrabrachial substance P infusion[14] and this release is sustained for at least 2 h.[15] In contrast, stimulation or antagonism of the endothelial ET_B receptor, with endothelin-1 and BQ-788 respectively, does not appear to influence forearm t-PA release. It is, therefore, unlikely that endothelin-1 provides a major contribution to the regulation of t-PA release in man, although we cannot exclude a small stimulatory effect.

Study limitations

In the forearm, typical resting arterio-venous differences are only ~10% of the total venous t-PA concentration and the basal constitutive release of t-PA antigen is ~0.9 ng/100 mL of tissue/min.[16] We have measured venous–venous differences between the infused and non-infused arms which, unlike the measurement of arterio–venous differences of the infused arm, has the disadvantage of not being able to correct for blood-flow-dependent changes in venous plasma t-PA concentrations. Theoretically (see Fig. 3), in the absence of an alteration in t-PA release, a 60% reduction in blood flow would be anticipated to increase total venous plasma t-PA concentrations by only ~7%, whereas a 200% increase in flow would reduce t-PA concentrations to the same degree (~7%). In the presence of stimulated t-PA release, these small flow-dependent changes are proportionately reduced even further.

The measurement of arterio–venous differences necessitates arterial sampling and the insertion of large-bore cannulae (19–20 gauge) which do not lend themselves to multiple cannulations within the same subject. Moreover, there is also the potential to introduce artefact from the presence of a larger thrombogenic surface, given that activated factor Xa is the most potent stimulant for t-PA release yet known.[28] To minimize arterial trauma and facilitate repeated studies in the same subjects, we have used 27-gauge arterial cannulae which permit drug infusion but not arterial blood sampling. However, we would suggest that flow-dependent changes in venous t-PA concentrations are small, within the variability of the t-PA assays (~5–7%) and are not of practical importance. Interestingly, a significant fall in the arterio–venous difference, or venous plasma concentration, of t-PA has not been detected during blood flow increases of up to 600% with sodium nitroprusside infusion.[14,16,29]

Measurement of venous–venous and arterio–venous differences both have the potential limitation that they can only estimate the net release of t-PA from the forearm and are unable to take account of clearance of t-PA within the forearm. However, the majority of t-PA is removed from the circulation by the liver[30] and the contribution of forearm clearance of t-PA is, therefore, likely to be very small.

During the present study, we did not see changes in heart rate or blood pressure to suggest systemic effects of endothelin-1[31] or BQ-788 infusion.[32] However, measuring venous concentrations bilaterally will control for any potential systemic effects which may go unrecognized if arterio–venous differences are measured in isolation. Once a drug has a systemic rather than a local effect, there is always the concern that subsequent t-PA release may be influenced or mediated by the release of other humoral factors, such as catecholamines. Moreover, if the main mechanism of t-PA release is mediated by a systemically released intermediate factor, then measuring arterio–venous differences could fail to detect this since arterial concentrations may remain unchanged and venous concentrations will rise in both forearms.

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The L-arginine/nitric oxide pathway contributes to the acute release of tissue plasminogen activator in vivo in man

David E. Newby [a,b,*], Robert A. Wright [b], Pamela Dawson [c], Christopher A. Ludlam [c], Nicholas A. Boon [b], Keith A.A. Fox [b], David J. Webb [a]

[a] *Clinical Pharmacology Unit and Research Centre, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK*
[b] *Department of Cardiology, University of Edinburgh, Royal Infirmary, Lauriston Place, Edinburgh EH3 9YW, UK*
[c] *Department of Haematology, University of Edinburgh, Royal Infirmary, Lauriston Place, Edinburgh EH3 9YW, UK*

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Abstract

Objective: Effective endogenous fibrinolysis requires rapid release of endothelial tissue plasminogen activator (t-PA). Using the nitric oxide synthase inhibitor, L-N^G-monomethylarginine (L-NMMA), we examined the contribution of endogenous nitric oxide to substance P-induced t-PA release in vivo in man. **Methods**: Blood flow and plasma fibrinolytic and haemostatic factors were measured in both forearms of 8 healthy male volunteers who received unilateral brachial artery infusions of substance P (2–8 pmol/min) and L-NMMA (1–4 μg/min). **Results**: Substance P caused dose-dependent increases in blood flow ($P < 0.001$) and plasma t-PA antigen ($P = 0.04$) and activity ($P < 0.001$) concentrations confined to the infused forearm, but had no effect on plasminogen activator inhibitor type 1 (PAI-1) or von Willebrand factor concentrations. In the presence of L-NMMA, substance P again caused significant increases in blood flow ($P < 0.001$) and t-PA antigen ($P = 0.003$) and activity ($P < 0.001$) concentrations but these increases were significantly less than with substance P alone ($P < 0.001$, $P = 0.05$ and $P < 0.01$, respectively). L-NMMA alone significantly reduced blood flow in the infused arm, but had no measurable effect on t-PA or PAI-1 concentrations. **Conclusions**: The L-arginine/nitric oxide pathway contributes to substance P-induced t-PA release in vivo in man. This provides an important potential mechanism whereby endothelial dysfunction increases the risk of atherothrombosis through a reduction in the acute fibrinolytic capacity.

Keywords: Thrombolysis; Endothelial factor; Nitric oxide; Blood flow; Endothelial function

1. Introduction

The endogenous fibrinolytic system can have important clinical effects as exemplified by the observation that in ~ 30% of patients with an acute myocardial infarction, the infarct-related artery spontaneously reperfuses within 12 h [1–3]. The ability of the endothelium to release tissue plasminogen activator (t-PA) rapidly is crucial if endogenous fibrinolysis within the arterial circulation is to be effective, with thrombus dissolution being much more effective if t-PA is incorporated during, rather than after, thrombus formation [4,5]. Epidemiological studies in a healthy male population and patients with ischaemic heart disease have shown a relationship between plasma fibrinolytic parameters and future cardiovascular events, such as stroke or myocardial infarction [6–9]. However, the capacity of endothelial cells to release t-PA from intracellular storage pools, and the rapidity with which this can be mobilised, may not be reflected in the basal circulating plasma concentrations of t-PA antigen or activity [10].

Endothelial cell culture techniques have limitations in the investigation of t-PA release and may not be truly representative of the in vivo function of these cells. The amount of t-PA released in culture is small and necessi-

* Corresponding author. Tel.: +44 (131) 332-1205; Fax: +44 (131) 343-6017; E-mail: d.e.newby@ed.ac.uk

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tates prolonged incubation periods and sensitive assays. Moreover, the phenotype of endothelial cells in culture, and the ability to release t-PA, changes with increasing passages. In contrast, under in vivo physiological conditions, the endothelium is arranged within a non-planar three-dimensional vascular bed, has a more favourable volume to surface area ratio, and is exposed to pulsatile blood flow and pressure changes. We have recently described an in vivo model to assess acute t-PA release in man [11]. Using intra-brachial infusions of substance P, we have shown a dose-dependent release of t-PA from the human forearm without causing significant release of von Willebrand factor (vWf) or plasminogen activator inhibitor type 1 (PAI-1). This suggests either a selective action of substance P or the lack of a rapidly translocatable pool of PAI-1 and vWf. However, we have previously used only brief (~ 10 min) substance P infusions [11] and protracted stimulation may release these factors [12–14].

Substance P causes endothelium dependent vasodilatation [15] which is mediated by the endothelial cell neurokinin type 1 receptor [16] and is, in part, related to the release of nitric oxide [17–19]. However, because t-PA release is not seen with infusions of the nitric oxide donor and vasodilator, sodium nitroprusside [11,20], an increase in nitric oxide and blood flow together do not release t-PA from the endothelium. Nevertheless, it remains a possibility that the L-arginine/nitric oxide pathway contributes to substance P-induced t-PA release.

Therefore, the aims of the current study were two-fold: first, to ascertain whether prolonged substance P infusion can cause vWf or PAI-1 release; and second, to determine whether nitric oxide synthase inhibition using L-N^G-monomethylarginine (L-NMMA) affects basal or substance P-induced t-PA release.

2. Methods

2.1. Subjects

Eight healthy men aged between 20 and 33 years participated in three studies which were 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. None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 h, and from food, tobacco and caffeine-containing drinks for at least 5 h, before each study. All studies were performed in a quiet, temperature-controlled room maintained at 23.5–24.5°C.

2.2. Intra-arterial administration and drugs

The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works, Birmingham, UK) under 1% lignocaine (Xylocaine; Astra Pharmaceuticals, Kings Langley, UK) local anaesthesia. The cannula was attached to a 16-gauge epidural catheter (Portex, Hythe, UK) and patency maintained by infusion of saline (0.9%; Baxter Healthcare, Thetford, UK) via an IVAC P1000 syringe pump (IVAC, Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 ml/min. Pharmaceutical-grade substance P (Clinalfa, Läufelfingen, Switzerland) and L-N^G-monomethylarginine (L-NMMA; Clinalfa) were administered following dissolution in saline.

2.3. Forearm blood flow and blood pressure

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges applied to the widest part of the forearm [21]. 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, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mmHg for 10 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 Strain Gauge Plethysmograph (D.E. Hokanson) was processed by a MacLab analogue-to-digital converter and Chart v3.3.8 software (AD Instruments, Castle Hill, Australia) and recorded onto a MacIntosh Classic II computer (Apple Computers, Cupertino, USA). Calibration was achieved using the internal standard of the plethysmograph.

Blood pressure was monitored in the non-infused arm at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer [22] (Takeda UA 751, Takeda Medical, Tokyo, Japan).

2.4. Venous sampling and assays

Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossa in both arms as described previously [23]. Ten ml of blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool Stabilyte, Umeå, Sweden; for t-PA assays) and citrate (Monovette, Sarstedt, Nümbrecht, Germany; for PAI-1 assays) tubes, and kept on ice before being centrifuged at 2000 *g* for 30 min at 4°C. Platelet-free plasma was decanted and stored at −80°C before assay.

Plasma PAI-1 and t-PA antigen concentrations were determined using an enzyme-linked immunosorbent assay (ELISA): Coaliza PAI-1 [24] and Coaliza t-PA [25] (Chromogenix AB, Mölndal, Sweden) respectively. Plasma PAI-1 and t-PA activities were determined by a photometric method, Coatest PAI-1 [26] and Coaset t-PA [27] (Chromogenix). 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 2.5 ng/ml, 0.5 ng/ml, 5 AU/ml and 0.10 IU/ml, respectively. vWf antigen was determined [28] using an ELISA (Dako, Glostrup, Denmark) with a sensitivity of 0.05 IU/ml. The intra-assay and inter-assay coefficients of variability were 5.2 and 7.3%, respectively. Factor VIII:C procoagulant activity was determined using a standard one-stage assay on an ACL-3000 + coagulometer (Instrumentation Laboratory, Warrington, UK). Haematocrit was determined by capillary tube centrifugation of blood anticoagulated by ethylene diamine tetraacetic acid and was obtained from the infused forearm at baseline and at 120 min.

2.5. Study design

On 3 separate occasions, at 09.00 h, subjects attended fasted and rested recumbent throughout each study. Strain gauges and cuffs were applied and the brachial artery of the non-dominant arm cannulated. Throughout all protocols, measurements of forearm blood flow were made every 10 min. Saline was infused for the first 30 min to allow time for equilibration and the final blood flow measurement during saline infusion was taken as the basal forearm blood flow. Thereafter, subjects underwent the following protocols, in random order, each separated by at least 1 week: protocol 1, each subject received intra-arterial substance P at 2, 4 and 8 pmol/min, for 10 min at each dose, followed by a continuous infusion of 8 pmol/min for a further 90 min; protocol 2, L-NMMA was co-infused at 4 μmol/min for 10 min before and throughout the same substance P infusion as protocol 1; and protocol 3, subjects received intra-arterial L-NMMA at 1, 2 and 4 μmol/min for 10 min at each dose followed by a continuous infusion of 4 μmol/min for a further 90 min. Venous samples were withdrawn from each arm at baseline and at 10, 20, 30, 50, 80 and 120 min after the start of substance P (for protocols 1 and 2) or L-NMMA infusion (protocol 3).

2.6. Data analysis and statistics

Plethysmographic data were extracted from the Chart data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel v4.0; Microsoft, Cambridge, USA). Recordings from the first 60 s after wrist-cuff inflation were not used because of the reflex vasoconstriction this causes [21]. Usually, the last five flow recordings in each 3-min measurement period were calculated and averaged for each arm. Estimated net release of t-PA activity and antigen was defined previously [11] as the product of the infused forearm plasma flow (based on the mean haematocrit, HCt, and the infused forearm blood flow, FBF) and the concentration difference between the infused ($[\text{t-PA}]_{\text{inf}}$) and non-infused arms ($[\text{t-PA}]_{\text{non-inf}}$).

Estimated net t-PA release

$$= \text{FBF} \times \{1 - \text{HCt}\} \times \{[\text{t-PA}]_{\text{inf}} - [\text{t-PA}]_{\text{non-inf}}\}$$

Data were examined, where appropriate, by two-way analysis of variance (ANOVA) with repeated measures and two-tailed paired Student's *t*-test using Excel v4.0 (Microsoft). Tachyphylaxis was assessed by comparing the 30-min (peak) and 120-min (final) values with a two-tailed paired Student's *t*-test. Area under the curve (AUC) was calculated for the estimated net release of t-PA across the study period. All results are expressed as mean ± s.e.m. Statistical significance was taken at the 5% level.

3. Results

All subjects were normotensive and there were no significant changes in blood pressure, heart rate or blood flow in the contralateral arm throughout any of the studies (Table 1). Haematocrit decreased slightly in each study (Table 1). Between the 3 protocols, there were no significant differences in the baseline values of blood pressure,

Table 1
Haemodynamics and haematocrit at baseline and completion of the 3 study protocols

	Substance P alone		L-NMMA alone		Substance P + L-NMMA	
	Basal	Final	Basal	Final	Basal	Final
Blood pressure (mmHg)						
Systolic	137 ± 3	136 ± 3	135 ± 5	136 ± 5	133 ± 6	132 ± 6
Diastolic	71 ± 2	71 ± 3	68 ± 3	71 ± 3	69 ± 4	70 ± 5
Heart rate (/min)	65 ± 4	61 ± 3	59 ± 2	58 ± 3	60 ± 3	61 ± 3
Absolute forearm blood flow (ml/100 ml/min)						
Non-infused arm	3.1 ± 0.3	3.7 ± 0.5	3.4 ± 0.7	3.6 ± 0.6	3.6 ± 0.5	4.9 ± 0.7
Infused arm	3.6 ± 0.4	12.1 ± 1.3[a]	3.9 ± 0.7	2.1 ± 0.2	4.2 ± 0.7	9.3 ± 1.4
Haematocrit	0.439 ± 0.006	0.430 ± 0.007[b]	0.430 ± 0.010	0.421 ± 0.013[c]	0.426 ± 0.008	0.414 ± 0.010[c]

[a] $P < 0.001$.
[b] $P < 0.005$.
[c] $P = 0.02$.

Table 2
Blood flow and plasma von Willebrand's factor and factor VIII:C activity concentrations in both arms during isolated substance infusion: protocol 1

	Baseline	Time (min)					
		10	20	30	50	80	120
Substance P dose (pmol/min)	0	2	4	8	8	8	8
Absolute forearm blood flow (ml/100 ml/min)							
Non-infused arm	3.1 ± 0.3	3.3 ± 0.3	3.4 ± 0.4	3.3 ± 0.3	3.5 ± 0.3	3.8 ± 0.4	3.7 ± 0.5
Infused arm	3.6 ± 0.4	11.8 ± 1.8	13.8 ± 1.9	15.9 ± 1.9	14.5 ± 1.7	12.6 ± 1.2	12.1 ± 1.3[a]
von Willebrand's factor (IU/ml)							
Non-infused arm	0.84 ± 0.13	0.64 ± 0.10	0.85 ± 0.13	0.60 ± 0.09	0.95 ± 0.18	0.80 ± 0.10	1.20 ± 0.16
Infused arm	0.73 ± 0.09	0.72 ± 0.15	0.73 ± 0.11	0.86 ± 0.20	0.98 ± 0.16	0.99 ± 0.17	1.03 ± 0.17
Factor VIII:C (IU/ml)							
Non-infused arm	0.50 ± 0.06	0.48 ± 0.04	0.51 ± 0.05	0.51 ± 0.05	0.58 ± 0.09	0.65 ± 0.10	0.72 ± 0.09
Infused arm	0.50 ± 0.05	0.50 ± 0.05	0.51 ± 0.07	0.52 ± 0.07	0.64 ± 0.07	0.64 ± 0.08	0.69 ± 0.10

[a] $P < 0.001$.

pendent manner (Fig. 1) reaching a maximum increase of 13.7 ± 1.7 ml/100 ml/min after 10 min at 8 pmol/min. This response underwent tachyphylaxis and decreased to 9.3 ± 1.4 ml/100 ml/min after 100 min of substance P at 8 pmol/min ($P < 0.002$ vs. 10 min). In comparison, with the non-infused arm, substance P co-infused with L-NMMA caused a dose-dependent increase in plasma t-PA activity ($P < 0.001$) and antigen ($P < 0.003$) concentrations of the infused arm which did not undergo significant tachyphylaxis (Fig. 2). L-NMMA caused a significant attenuation of substance P-induced increases in blood flow ($P < 0.001$) and plasma t-PA activity concentrations ($P < 0.003$) in the infused forearm, but not plasma t-PA antigen.

3.3. Estimated net t-PA production

L-NMMA infused alone had no significant effects on t-PA release: 95% confidence intervals for t-PA antigen and activity release are 0.31 to −0.68 ng/100 ml/min and 0.27 to −0.06 IU/100 ml/min, respectively. Sub-

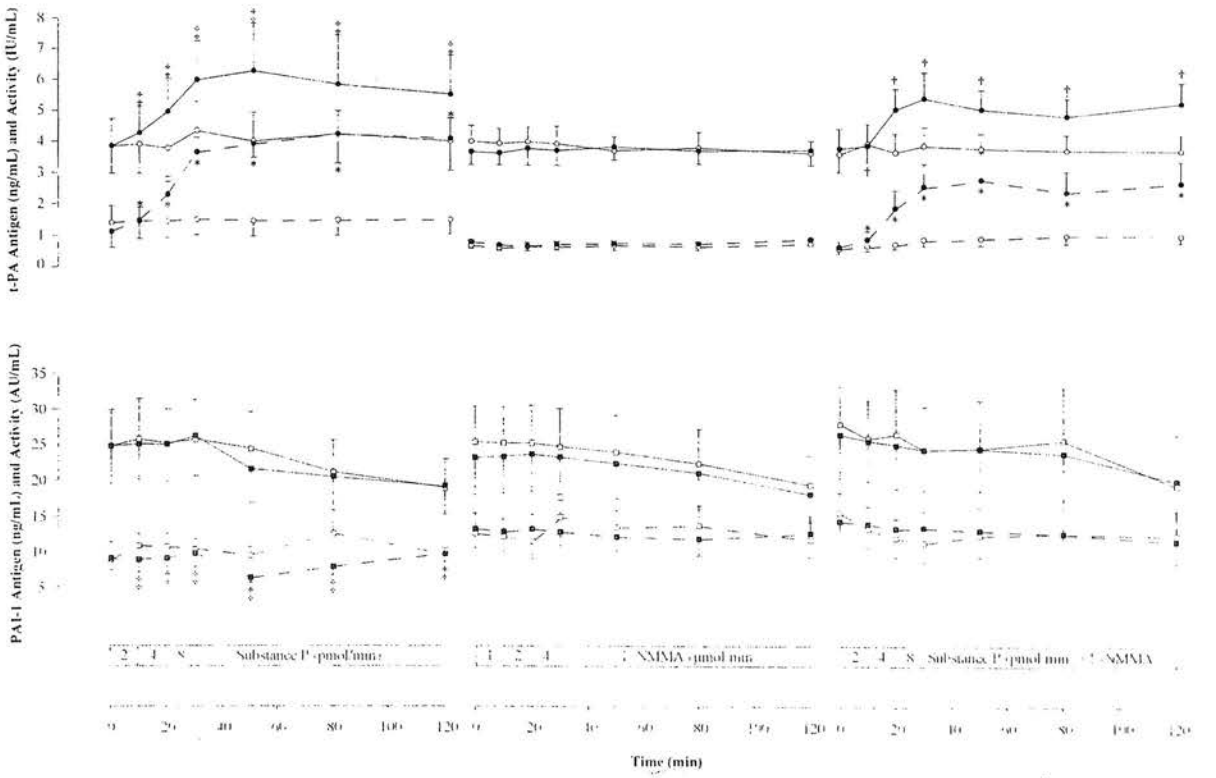

Fig. 2. Plasma concentrations of t-PA and PAI-1 antigen (solid lines) and activity (dashed lines) in the infused (● and ■, respectively) and non-infused (○ and □, respectively) arms in the 3 protocols. $P < 0.001$; $P < 0.003$; $P < 0.04$ (ANOVA).

heart rate, forearm blood flow, haematocrit or plasma concentrations of t-PA and PAI-1 antigen and activity.

3.1. Isolated infusions of substance P and L-NMMA

Substance P increased blood flow in the infused arm ($P < 0.001$) in a dose-dependent manner (Fig. 1 and Table 2) reaching a maximum increase of 15.9 ± 1.9 ml/100 ml/min after 10 min at 8 pmol/min. Following prolonged infusion, substance P-induced vasodilatation demonstrated tachyphylaxis and decreased to 12.1 ± 1.3 ml/100 ml/min after 100 min of substance P at 8 pmol/min ($P < 0.003$ vs. 10 min). In comparison to the non-infused arm, substance P caused a dose-dependent increase in venous plasma t-PA activity ($P < 0.001$) and antigen ($P < 0.04$) concentrations of the infused arm which did not undergo significant tachyphylaxis (Fig. 2). Concentrations of plasma PAI-1 activity were also reduced in the infused arm ($P = 0.04$; Fig. 2). In contrast, there were no significant changes in plasma PAI-1 antigen, vWf or factor VIII:C concentrations in either arm (Fig. 2 and Table 2).

L-NMMA decreased blood flow in the infused arm ($P < 0.001$) in a dose-dependent manner (Fig. 1 and Table 2) reaching 2.1 ± 0.2 ml/100 ml/min after 100 min at 4 μmol/min. There were no significant changes in the concentrations of plasma t-PA and PAI-1 antigen or activity in either arm during infusion of L-NMMA (Fig. 2).

3.2. Co-infusion of L-NMMA and substance P

In the presence of L-NMMA, substance P increased blood flow in the infused arm ($P < 0.001$) in a dose-de-

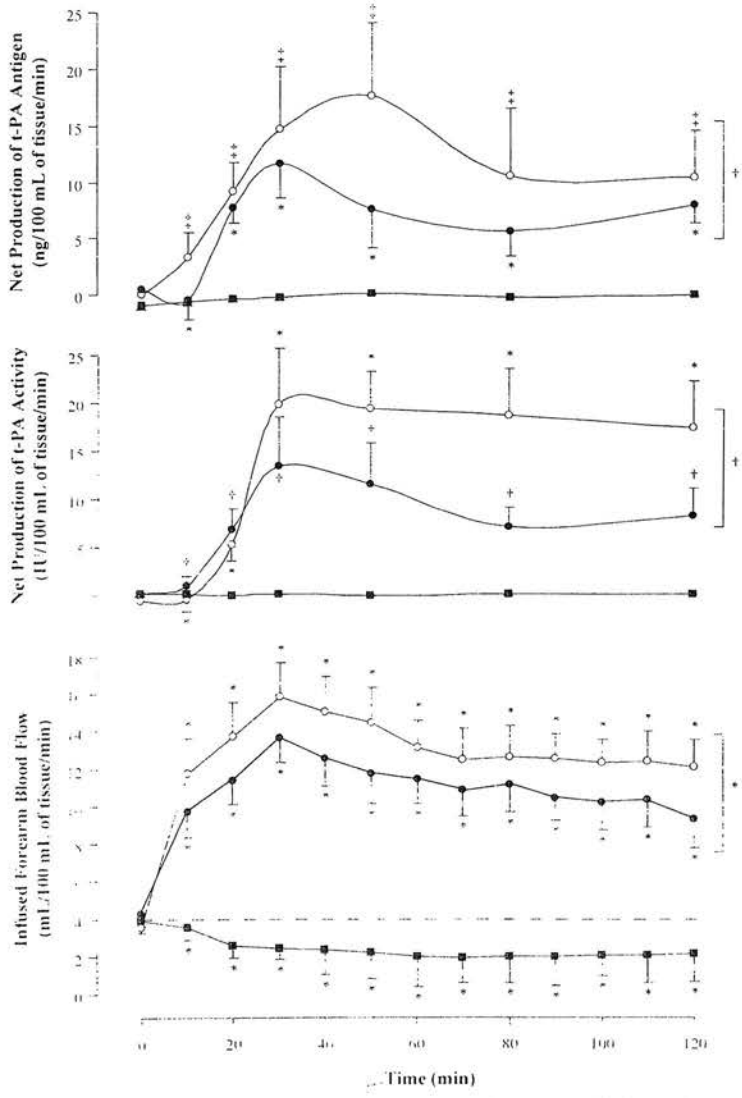

Fig. 1. Infused forearm blood flow and estimated net release of t-PA antigen and activity during protocol 1 (substance P alone, ○), protocol 2 (substance P and L-NMMA, ●) and protocol 3 (L-NMMA alone; ■). $^{\dagger}P < 0.001$; $^{*}P \leq 0.05$; $P = 0.09$ (ANOVA).

stance P caused dose-dependent increases in the estimated net release of t-PA antigen and activity in the presence or absence of L-NMMA ($P < 0.001$) which did not undergo significant tachyphylaxis. However, the magnitude of the increase in release of both t-PA antigen ($P = 0.05$) and activity ($P < 0.01$) was significantly reduced in the presence of L-NMMA (Fig. 1). L-NMMA reduced the AUC for the substance P-induced release of t-PA antigen and activity by 40 and 46% respectively.

4. Discussion

We have shown that intra-brachial substance P infusion increases forearm blood flow and plasma t-PA concentrations for up to 2 h without a demonstrable effect on plasma PAI-1 or vWf concentrations. Although the nitric oxide synthase inhibitor, L-NMMA, significantly reduced forearm blood flow without affecting basal t-PA release, it inhibited the increases in blood flow, plasma t-PA concentrations and t-PA release produced by substance P administration in the forearm. These data suggest that the L-arginine/nitric oxide pathway contributes to substance P-induced t-PA release in vivo in man. In contrast, we [11] and others [20] have shown previously that t-PA release is not seen with the large local increases in nitric oxide delivery and blood flow associated with infusions of the nitric oxide donor, sodium nitroprusside. Taken together, these findings indicate that increases in nitric oxide and blood flow are not sufficient per se to release t-PA but, through the L-arginine/nitric oxide pathway, are able to enhance substance P-induced t-PA release.

The permissive role of intracellular mediators in the mechanism of t-PA release has been described previously. In the rat perfused hindlimb model, increasing intracellular calcium alone is insufficient to cause t-PA release whilst it is essential for bradykinin induced t-PA release [29]. However, the regulation of t-PA release is complex and may involve several signal transduction pathways [30]. This is reflected by the diversity of mediators, such as thrombin, bradykinin and desmopressin, which can release t-PA and increase t-PA activity [12,13,31,32]. One can, therefore, only speculate as to whether our findings extend to the acute t-PA release seen with in situ thrombosis. However, both nitric oxide mediated endothelial dysfunction [33–36] and abnormalities of endogenous fibrinolysis [6–9] have been described in many atherosclerotic diseases and the associated risk factors. Thus, the coupling of acute t-PA release to the L-arginine/nitric oxide pathway provides an important potential mechanism whereby endothelial dysfunction might increase the risk of atherothrombosis through a reduction in the acute fibrinolytic capacity. Our initial findings would suggest that this model could be applied to the assessment of the acute fibrinolytic capacity of patients with endothelial dysfunction such as those with hypercholesterolaemia and a smoking habit [35,36], and to the examination of the subsequent effect of L-arginine supplementation.

Substance P-induced vasodilatation undergoes tachyphylaxis [37] which may relate to internalisation of the neurokinin type 1 receptor from the endothelial cell surface membrane [38]. It has been suggested from ex vivo animal studies [15,39] that the residual vasodilatation following the development of tachyphylaxis is almost completely nitric oxide-dependent. In the present study, the degree of inhibition of substance P-induced vasodilatation by L-NMMA was less than we [18] and others [17] have previously described and may reflect the higher potency and doses used in this study. Whilst we have readily demonstrated tachyphylaxis of substance P-induced vasodilatation, the co-infusion of L-NMMA did not affect the development of tachyphylaxis and did not abolish the residual substance P-induced vasodilatation following its development. Thus, in contrast to animal studies, residual vasodilatation after the development of tachyphylaxis does not appear to be predominantly nitric oxide mediated in the human forearm. In addition, we were unable to detect significant tachyphylaxis of substance P-induced increases in plasma t-PA antigen and activity concentrations, suggesting that not all the actions of substance P undergo tachyphylaxis.

The substance P-induced reductions in plasma PAI-1 activity of the infused arm without significant alterations in PAI-1 antigen concentrations are consistent with acute t-PA release in the absence of PAI-1 release [40]. PAI-1 binds to the newly released t-PA to form an inactive PAI-1/t-PA complex, thereby reducing the plasma PAI-1 activity. The trend for PAI-1 antigen concentrations to fall in both arms as the study progressed is consistent with systemic (hepatic) clearance of the PAI-1/t-PA complex [40–42]. However, this trend was also seen with isolated L-NMMA infusion in which there was no significant release of t-PA consistent with a circadian fall of PAI-1 antigen during the morning [43].

Despite reducing forearm blood flow by half, L-NMMA did not significantly affect the constitutive release or plasma concentrations of t-PA and PAI-1 antigen and activity. The 95% confidence intervals indicates that if L-NMMA has an effect on basal t-PA or PAI-1 release then it is rather small. This suggests that the L-arginine/nitric oxide pathway does not play a major role in the basal release of t-PA or PAI-1 in the peripheral vasculature of man.

4.1. Study limitations

Since the derivation of t-PA release is a function of plasma flow, it could be argued that the inhibition by L-NMMA of substance P-induced t-PA release reflects the simultaneous reduction in blood flow. However, the reduction in absolute blood flow was only modest (15–20%) in comparison to the reduction in t-PA release (40–46%) and the plasma t-PA activity concentrations in the infused

forearm were also significantly reduced by co-infusion of L-NMMA. The findings of the present study would be strengthened by utilising a control vasoconstrictor and demonstrating a neutral effect on substance P-induced t-PA release. However, standard receptor coupled vasoconstrictors used in forearm studies, such as noradrenaline, vasopressin and angiotensin II, are known to stimulate t-PA and PAI-1 release [12,14,32,44] and would not help in interpreting the influence of L-NMMA on substance P-induced t-PA release.

We have previously been unable to detect an acute local release of either vWf or PAI-1 during 10-min infusions of substance P given at 8-fold higher concentrations [11]. In the present study, substance P did not cause significant vWf or PAI-1 release, despite infusion times of up to 120 min, suggesting that the dissociation of substance P-induced t-PA release from vWf is not a temporal effect. However, this dissociated release does not appear to be unique to substance P since this has also been recently described with local forearm infusions of desmopressin [45]. These findings are, however, limited to the peripheral forearm vascular bed and the endothelium in other tissue beds may respond differently to substance P stimulation. The extension of this model to vascular beds associated with atherosclerosis such as the coronary circulation, will be of crucial relevance in determining the influence of atheroma and endothelial dysfunction on the acute local release of t-PA during thrombotic occlusion and plaque rupture.

In summary, in the forearm vascular bed in vivo, we have shown for the first time that the L-arginine/nitric oxide pathway contributes to substance P-induced t-PA release in man. This coupling of acute t-PA release to the L-arginine/nitric oxide pathway provides an important potential mechanism whereby endothelial dysfunction increases the risk of atherothrombosis through a reduction in the acute fibrinolytic capacity.

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Endothelial Dysfunction, Impaired Endogenous Fibrinolysis, and Cigarette Smoking

A Mechanism for Arterial Thrombosis and Myocardial Infarction

David E. Newby, BA, BSc, BM, MRCP; Robert A. Wright, MB, ChB, MRCP;
Catherine Labinjoh, BSc, MB, ChB, MRCP; Christopher A. Ludlam, PhD, FRCP, FRCPath;
Keith A.A. Fox, BSc, MB, ChB, FRCP, FESC;
Nicholas A. Boon, MD, FRCP; David J. Webb, MD, FRCP, FRCPE, FFPM

Background—Effective endogenous fibrinolysis requires rapid release of tissue plasminogen activator (tPA) from the vascular endothelium. Smoking is a known risk factor for arterial thrombosis and myocardial infarction, and it causes endothelial dysfunction. We therefore examined the effects of cigarette smoking on substance P–induced tPA release in vivo in humans.

Methods and Results—Blood flow and plasma fibrinolytic factors were measured in both forearms of 12 smokers and 12 age- and sex-matched nonsmokers who received unilateral brachial artery infusions of substance P (2 to 8 pmol/min). In both smokers and nonsmokers, substance P caused dose-dependent increases in blood flow and local release of plasma tPA antigen and activity ($P<0.001$ for all) but had no effect on the local release of plasminogen activator inhibitor type 1. Compared with nonsmokers, increases in forearm blood flow ($P=0.03$) and release of tPA antigen ($P=0.04$) and activity ($P<0.001$) caused by substance P were reduced in smokers. The area under the curve for release of tPA antigen and activity decreased by 51% and 53%, respectively.

Conclusions—Cigarette smoking causes marked inhibition of substance P–induced tPA release in vivo in humans. This provides an important mechanism whereby endothelial dysfunction may increase the risk of atherothrombosis through a reduction in the acute fibrinolytic capacity. (***Circulation.* 1999;99:1411-1415.**)

Key Words: plasminogen activators ■ endothelium ■ endothelium-derived factors ■ blood flow

Acute rupture of a coronary atheromatous plaque and subsequent coronary artery thrombosis causes the majority of sudden cardiac deaths and myocardial infarctions.[1,2] Cigarette smoking not only is strongly associated with atherosclerosis[3] and ischemic heart disease[4] but also is a major risk factor for acute coronary thrombosis.[1,5] Indeed, 75% of sudden cardiac deaths due to acute thrombosis are in cigarette smokers.[1] Smoking causes endothelial dysfunction[6] and is associated with increased platelet thrombus formation.[5] Small areas of denudation and thrombus deposition are a common finding on the surface of atheromatous plaques[7,8] and are usually subclinical. However, in the presence of an imbalance in the coagulation or fibrinolytic systems, such microthrombi may propagate, ultimately leading to arterial occlusion.

The importance of endogenous tissue plasminogen activator (tPA) release is exemplified by the high rate of spontaneous reperfusion in the infarct-related artery after acute myocardial infarction, occurring in ≈30% of patients within the first 12 hours.[9–11] It would be anticipated that high plasma tPA concentrations should protect against subsequent coronary events. However, in epidemiological studies of patients with ischemic heart disease[12,13] and in a healthy male population (US Physicians Study),[14] higher total plasma tPA (antigen) concentrations positively predict future coronary events. This is explained by the concomitant elevation of plasminogen activator inhibitor type 1 (PAI-1), which forms a complex with tPA and thereby causes an overall reduction in free tPA "activity."[15,16] It is this free and unbound tPA that is physiologically active and leads to endogenous fibrinolysis. However, the capacity of endothelial cells to release tPA from intracellular storage pools and the rapidity with which this can be mobilized may not necessarily be reflected in the basal circulating plasma concentrations of tPA antigen or activity.[17]

Using the endothelium-dependent vasodilator substance P to stimulate tPA release, we recently described an in vivo model to assess the acute fibrinolytic capacity of the human

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From the Clinical Pharmacology Unit and Research Centre, University of Edinburgh, Western General Hospital (D.E.N., C.L., D.J.W.), and the Departments of Cardiology (D.E.N., R.A.W., C.L., K.A.A.F., N.A.B.) and Haematology (C.A.L.), University of Edinburgh, Royal Infirmary, Edinburgh, Scotland, UK. Dr Wright is now at the Department of Cardiology, The Ayr Hospital, Ayr, Scotland, UK.
Correspondence to Dr D.E. Newby, Clinical Pharmacology Unit and Research Centre, University of Edinburgh, Western General Hospital, Crewe Rd, Edinburgh EH4 2XU, Scotland, UK. E-mail d.e.newby@ed.ac.uk

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forearm.[18] Moreover, we have been able to demonstrate a reduction in tPA release after inducing experimental "endothelial dysfunction" with nitric oxide synthase inhibition.[19] We therefore hypothesized that cigarette smoking might impair endogenous fibrinolysis by reducing the capacity of the endothelium to release tPA acutely. The aim of the study was to compare substance P–induced tPA release from the forearm vascular bed of smokers and age- and sex-matched nonsmokers.

Methods

Subjects

Twelve healthy smokers (5 to 20 cigarettes/d) and 12 age- and sex-matched nonsmokers between 25 and 55 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.

All subjects were normotensive without a history of diabetes mellitus or vascular disease. Female subjects were premenopausal and not receiving hormonal contraceptives. They were clinically well and taking no regular medications. 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' standing and maintained their normal smoking habits in the week before attendance. None of the subjects received vasoactive or nonsteroidal anti-inflammatory drugs in the week before the study, and all abstained from alcohol for 24 hours before and from food, tobacco, and caffeine-containing drinks on the day of the study. All studies were performed in a quiet, temperature-controlled room maintained at 23.5°C to 24.5°C.

Intra-Arterial Drug Administration

The brachial artery of the nondominant arm was cannulated with a 27–standard wire gauge steel needle (Cooper's Needle Works Ltd) under local anesthesia. The cannula was attached to a 16-gauge epidural catheter (Portex Ltd), and patency was maintained by infusion of saline (0.9%: Baxter Health Care Ltd) via an IVAC P1000 syringe pump (IVAC Ltd). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 mL/min. Pharmaceutical-grade substance P (Clinalfa AG) was administered after dissolution in saline.

Measurements

Blood flow was measured in both forearms by venous occlusion plethysmography as previously described.[18–20] Blood pressure was monitored in the noninfused arm at intervals throughout each study with a semiautomated noninvasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc).

Venous cannulas (17-gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms. Blood (10 mL) was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool Stabilyte, for tPA assays) and citrate (Monovette, for PAI-1 assays) 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. Plasma PAI-1 and tPA antigen and activities were determined as previously described[18,19] with an ELISA (Coaliza PAI-1 and Coaliza tPA, Chromogenix AB) and a photometric method (Coatest PAI-1 and Coaset tPA, Chromogenix AB). Hematocrit was determined by capillary tube centrifugation at baseline and during infusion of 8 pmol/min of substance P. Plasma lipid fractions were measured by an enzymatic colorimetric method (Boehringer Mannheim GmbH Diagnostica). LDL cholesterol was derived according to the method of Friedewald et al.[21]

TABLE 1. Baseline Subject Characteristics

	Nonsmokers	Smokers
Age, y	35±3	34±2
Sex, male:female	10:2	10:2
Body mass index, kg/m^2	23.9±0.5	24.5±1.1
Mean arterial pressure, mm Hg	92±2	91±2
Heart rate, bpm	59±3	66±2
Fasting plasma glucose, mmol/L	5.3±0.1	5.4±0.1
Total cholesterol, mg/dL	189±14	200±16
LDL cholesterol, mg/dL	113±13	130±16
HDL cholesterol, mg/dL	48±3	37±2*
Triglycerides, mg/dL	138±16	163±27
Baseline hematocrit	0.41±0.01	0.43±0.01

*P=0.01 (unpaired t test, smokers vs nonsmokers).

Study Design

At 9 AM, subjects attended fasted and then rested recumbent throughout each study. Strain gauges and cuffs were applied, and the brachial artery of the nondominant arm was cannulated. Forearm blood flow was measured every 10 minutes. Saline was infused for the first 30 minutes to allow time for equilibration. The final blood flow measurement during saline infusion was taken as the basal forearm blood flow. Thereafter, subjects received intra-arterial substance P at 2, 4, and 8 pmol/min for 10 minutes at each dose.

Data Analysis and Statistics

This study's population size, on the basis of power calculations derived from previous studies, gives 90% power of detecting an 18% difference in tPA release at a significance level of 5%. Coefficients of repeatability[22] for plasma concentrations of tPA antigen and activity during substance P infusion at 8 pmol/min are 1.6 ng/mL and 1.4 IU/mL, respectively (data on file).

Plethysmographic data were extracted from the Chart data files, and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel version 5.0; Microsoft Corp). Recordings from the first 60 seconds after wrist cuff inflation were not used because of the reflex vasoconstriction this causes.[20] Usually, the last 5 flow recordings in each 3-minute measurement period were calculated and averaged for each arm. Estimated net release of tPA activity and antigen was defined previously[18] as the product of the infused forearm plasma flow (based on the mean hematocrit, Hct, and the infused forearm blood flow, FBF) and the concentration difference between the infused ($[tPA]_{Inf}$) and noninfused ($[tPA]_{Noninf}$) arms: Estimated net tPA release$=FBF\times(1-Hct)\times([tPA]_{Inf}-[tPA]_{Noninf})$.

Data were examined, where appropriate, by 2-way ANOVA with repeated measures and 2-tailed Student's t test using Excel version 5.0 (Microsoft). The area under the curve was calculated for the estimated net release of tPA across the study period. All results are expressed as mean±SEM. Statistical significance was taken at the 5% level.

Results

There were no significant differences in baseline characteristics, except that smokers had a slightly lower HDL concentration (Table 1). There were no significant changes in blood pressure, heart rate, hematocrit, or blood flow in the noninfused forearm during the study (data on file; Table 2). In the noninfused arm, plasma tPA antigen concentrations were higher in smokers than nonsmokers (P=0.02; Table 2). There were no significant differences in plasma PAI-1 antigen and activity between the groups.

TABLE 2. Blood Flow and Plasma tPA and PAI-1 Antigen and Activity Concentrations in Both Forearms

	Nonsmokers				Smokers			
		Time, min				Time, min		
	Baseline	10	20	30	Baseline	10	20	30
Substance P dose, pmol/min	0	2	4	8	0	2	4	8
Absolute forearm blood flow, mL · 100 mL^{-1} · min^{-1}								
Noninfused arm	2.8±0.3	2.9±0.4	2.9±0.4	2.8±0.3	2.8±0.2	2.9±0.3	2.9±0.3	3.0±0.3
Infused arm	3.7±0.4	11.2±1.1	13.5±1.3	16.2±1.5*	3.6±0.3	9.4±0.4	11.5±0.7	14.2±0.8*†
tPA antigen, ng/mL								
Noninfused arm	3.3±0.5	3.4±0.5	3.4±0.5	3.7±0.5	4.0±0.5	4.3±0.5	4.4±0.5	4.4±0.6†
Infused arm	3.2±0.5	4.1±0.6	4.4±0.6	6.2±0.8*	4.1±0.5	4.5±0.6	5.2±0.7	5.9±0.9*
tPA activity, IU/mL								
Noninfused arm	0.8±0.2	0.9±0.2	1.0±0.2	1.3±0.2	0.7±0.1	0.7±0.1	0.8±0.1	1.0±0.2
Infused arm	0.8±0.2	2.1±0.5	2.8±0.5	4.6±0.6*	0.7±0.1	1.1±0.2	1.7±0.4	3.0±0.5*‡
PAI-1 antigen, ng/mL								
Noninfused arm	29±7	29±6	28±7	28±6	29±6	26±5	25±5	26±5
Infused arm	28±6	28±7	27±6	28±5	26±5	27±6	26±6	26±5
PAI-1 activity, AU/mL								
Noninfused arm	11.8±1.7	11.8±1.7	12.1±1.6	11.4±1.8	12.0±2.0	11.0±1.7	9.2±1.4	10.2±1.3
Infused arm	10.7±1.6	8.8±1.5	10.8±1.7	9.3±1.5	12.5±1.9	10.6±1.5	10.5±1.2	8.5±1.1

One-way ANOVA: *$P<0.001$; 2-way ANOVA (nonsmokers vs smokers): †$P<0.05$; ‡$P=0.001$.

Substance P caused dose-dependent increases in forearm blood flow in the infused arm in both smokers and nonsmokers (Table 2, Figure), but the increase in blood flow was greater in nonsmokers ($P=0.03$; 2-way ANOVA, nonsmokers versus smokers). Compared with the noninfused arm (2-way ANOVA), substance P caused dose-dependent increases in plasma concentrations of tPA antigen ($P<0.001$) and activity ($P<0.001$) in the infused arm of both smokers and nonsmokers (Table 2). There were no significant changes in plasma PAI-1 antigen or activity in either group. The increase in plasma tPA activity in the infused arm was greater in the nonsmokers ($P=0.001$; 2-way ANOVA, nonsmokers versus smokers).

Substance P increased the net release of tPA antigen ($P=0.009$) and activity ($P<0.001$) in smokers (Figure). In nonsmokers, substance P increased the net release of tPA antigen ($P<0.001$) and activity ($P<0.001$) significantly more than in smokers ($P=0.04$ and $P<0.001$, respectively; 2-way ANOVA, nonsmokers versus smokers). Compared with the nonsmokers, the area under the curve for net tPA antigen and activity release was reduced by 51% and 53%, respectively, in the smokers.

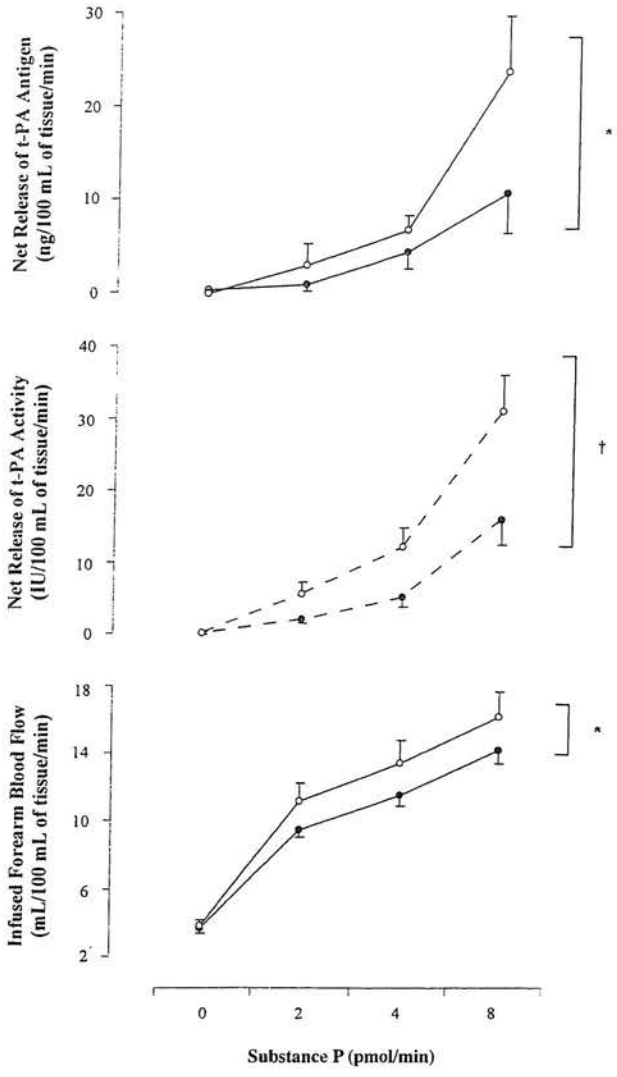
Subgroup analysis after exclusion of female subjects did not alter the magnitude or the statistical significance of the above findings. Qualitatively, the responses in female smokers and nonsmokers were similar to those observed in the male subjects.

Discussion

We have shown here, for the first time, that despite higher basal plasma tPA antigen concentrations, cigarette smokers have a markedly impaired capacity of the endothelium to release tPA acutely. This establishes an important mechanism whereby cigarette smoking can lead to arterial thrombosis and myocardial infarction.

The rapid mobilization of tPA from the endothelium is crucial if endogenous fibrinolysis within the arterial circulation is to be effective, with thrombus dissolution being much more effective if tPA is incorporated during, rather than after, thrombus formation.[23,24] The increased risk of spontaneous thrombosis seen in smokers may therefore plausibly relate to the propagation of thrombus, which would otherwise undergo lysis and remain subclinical. Although cigarette smokers have a higher overall mortality from myocardial infarction than nonsmokers,[25] the in-hospital mortality is lower.[26–28] This apparent paradox can be explained by the observation that the infarct-related artery is more than twice as likely to become patent in current smokers as in nonsmokers after thrombolytic therapy for acute myocardial infarction.[28–30] Indeed, it has been suggested[30] that thrombolytic therapy should only be given to smokers and that alternative strategies such as primary angioplasty should be used in nonsmokers. These observations are consistent with the present findings because it might be anticipated that patients with impaired endothelial cell tPA release would benefit most from thrombolytic therapy, whereas those with a normal endogenous fibrinolytic capacity are more likely to have tPA-resistant thrombus, which would respond less favorably.

Our findings in smokers are consistent with the previous observational data[12–14] that increased basal plasma concentrations of tPA antigen are associated with future coronary events. The assessment of endogenous fibrinolysis has previously relied on measurement of basal plasma tPA concentrations and the acute release of tPA in response to venous



Infused forearm blood flow and the net release of tPA antigen and activity in smokers (●) and nonsmokers (○). $P<0.001$ (1-way ANOVA) for all responses. Two-way ANOVA (nonsmokers vs smokers): *$P<0.05$; †$P<0.001$.

occlusion, systemic desmopressin infusion, or exercise.[31–34] However, because of confounding systemic effects and the nonuniformity of the stimuli applied, these responses can be variable and give only a relatively crude measure of fibrinolytic capacity. Moreover, although it has previously been shown that systemic desmopressin infusion causes less tPA release in smokers,[31] this effect may not be directly endothelium-dependent.[35] In contrast, we have used locally active doses of substance P to provide a more precise pharmacological stimulus to the endothelium and to cause a substantial and dose-dependent local release of tPA.[18,19] This has allowed us to demonstrate a distinct and marked inhibition of stimulated endothelial tPA release in smokers.

Although thrombin is more physiologically relevant to acute tPA release than substance P, we have used the latter because its vascular actions are endothelium-dependent,[36] mediated in part through nitric oxide,[37] and its administration intra-arterially is safe and well tolerated.[38] Consistent with previous workers,[6,39,40] we have also found an attenuation of the endothelium-dependent forearm blood flow responses in smokers. This inhibition of both the blood flow and tPA response may, in part, relate to an impairment of the L-arginine:nitric oxide pathway in smokers.[19,39] Although differences exist,[41] the forearm model may provide a useful surrogate for the coronary vascular bed[42,43] and permits a readily accessible and reliable assessment of endothelial cell function. However, the present findings need to be confirmed in the coronary circulation.

We have studied the sustained effect of chronic smoking in a selected healthy and predominantly male population at a single time point. Although total and LDL cholesterol concentrations were similar in smokers and nonsmokers, HDL cholesterol concentrations were slightly lower in smokers. This is not unexpected, because cigarette smoking is known to be associated with a selective reduction in HDL cholesterol concentrations.[44,45] However, the application of this model to other conditions associated with endothelial dysfunction, such as dyslipidemia, is warranted. Finally, because hormonal status influences fibrinolytic parameters,[46] the assessment of the acute fibrinolytic capacity in premenopausal and postmenopausal women and the modulating effect of hormonal therapy will also be of particular interest.

In conclusion, we have demonstrated a major impairment of tPA release from the vascular endothelium of smokers. Our findings suggest that the fundamental mechanism whereby cigarette smoking causes arterial thrombosis and myocardial infarction relates, at least in part, to impairment of the acute endogenous fibrinolytic capacity.

Acknowledgments

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