Endothelial Function and Endogenous Fibrinolysis in Inflammation and Ischaemic Heart Disease

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I declare that,

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ABSTRACT OF THESIS

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The vascular endothelium plays a vital role in the control of blood flow, haemostasis, fibrinolysis and inflammation. Impairment of endothelial vasomotor and fibrinolytic function is implicated in the pathogenesis of ischaemic heart disease. Atherosclerosis is now widely recognised to be an inflammatory disease process, but the mechanistic links between inflammation, endothelial dysfunction and endogenous fibrinolysis remain poorly understood.

In a series of studies, we explored the effects of inflammation on endothelial vasomotion and fibrinolytic capacity using an *in vivo* forearm model of systemic and local inflammation. Systemic inflammation stimulated by typhoid vaccination had no major effect on vasomotor tone. However, tumour necrosis factor-α induced local vascular inflammation was associated with impaired resistance vessel endothelium-dependent vasodilatation, possibly through the development of acute arterial injury. Both systemic and local inflammation were found to augment the acute release of endothelial tissue plasminogen activator. In addition, intra-arterial tumour necrosis factor-α administration resulted in a unique profile of substantial and sustained local increase in endogenous tissue plasminogen activator.

We extended these investigations and assessed the role of endogenous fibrinolysis and endothelial dysfunction in the pathogenesis of prothrombotic conditions such as hyperhomocysteinaemia and coronary stent thrombosis or in-stent restenosis using this forearm model of endothelial function assessment. In patients with recent myocardial infarction, elevation of plasma homocysteine concentration was associated with impaired endothelium-dependent vasodilatation but not endogenous fibrinolysis. This vasomotor dysfunction was not rectified by vitamin supplementation. We also assessed three critical aspects of vascular function in patients who have undergone percutaneous coronary intervention and found no evidence that endothelial vasomotor, fibrinolytic or platelet function play a major role in the pathogenesis of acute stent thrombosis or in-stent restenosis.

We conclude that there is complex interaction between inflammation, endothelial function and endogenous fibrinolysis. We have identified the unique role of systemic inflammation and specifically, tumour necrosis factor-α. Furthermore, our studies indicated that there were no significant associations between fibrinolysis and hyperhomocysteinaemia or coronary stent complications. Therefore modulating cytokine actions and their interaction with fibrinolysis may be critical in the prevention of thrombotic coronary occlusion and myocardial ischaemia as well as in the future development of anti-thrombotic therapies.

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Abbreviations

ADP	adenosine diphosphate
ANOVA	analysis of variance
AUC	area under the curve
CRP	C-reactive protein
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
FBF	forearm blood flow
Hct	haematocrit
IL-1	interleukin-1
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
PAI-1	plasminogen activator inhibitor type 1
PAI-2	plasminogen activator inhibitor type 2
PCI	percutaneous coronary intervention
TNF-α	tumour necrosis factor-α
t-PA	tissue-type plasminogen activator
u-PA	urokinase-type plasminogen activator

Figure Legend

Table Legend

Chapter 1

Introduction:

Endothelial Function, Endogenous Fibrinolysis and Inflammation

1.1 Vascular Endothelium

1.1.1 Normal Endothelial Function

The endothelium is a single–cell lining covering the internal surface of the circulatory system. Initially regarded as a relatively inert semipermeable barrier between blood and vessel wall, it is now recognised to have an enormous range of crucial homeostatic functions and participates in metabolic, synthetic and regulatory pathways [Cines *et al* 1998]. Strategically located at the interface between tissue and blood, the vascular endothelium regulates the flow of nutrient substances, diverse biologically active molecules and blood cells, and plays a key role in the control of vascular tone, local coagulation and fibrinolysis, inflammation and vascular proliferative processes. By means of membrane receptor mechanisms, the endothelium is able to detect and respond to physical and chemical stimuli by the release of a variety of vasoactive and thromboregulatory molecules, that include nitric oxide, endothelins, interleukins, plasminogen activators and inhibitors, and von Willebrand factor.

Under normal conditions, the effects of these endothelial factors maintain normal vascular tone, blood fluidity and limit vascular inflammation and smooth muscle cell proliferation. However, in the presence of coronary risk factors, endothelial function may be impaired and predispose the vasculature to alterations in vascular tone, leukocyte adherence, platelet activation, vascular inflammation, thrombosis and atherosclerotic lesion formation [Ross 1999].

1.1.2 Regulation of Vasomotor Tone

Following the seminal work of Furchgott and Zawadski, it has been widely recognised that an array of mediators can influence vascular tone through endothelium-dependent actions [Furchgott & Zawadski 1980]. Vasodilators such as nitric oxide and prostacyclin, as well as vasoconstrictors, including endothelin-1 and platelet activating factor, are secreted by the vascular endothelium to maintain and regulate tissue perfusion. Endothelial cells may also participate in vasoregulation following stimulation by circulating vasodilators and constrictors, such as thrombin, bradykinin and adenosine diphosphate.

1.1.3 Endothelium-dependent Vasodilators

Nitric oxide is a key endothelium-derived relaxing factor. A heterodiatomic free radical product, nitric oxide is generated through the oxidation of L-arginine to L-citrulline by nitric oxide synthase [Palmer *et al* 1988; Stamler *et al* 1992]. One isoform, endothelial nitric oxide synthase (eNOS), is constitutively expressed and active in endothelial cells, but can be upregulated by receptor-dependent agonists. Nitric oxide tonically relaxes arterial vessels at rest in both the systemic and pulmonary circulation [Vallance *et al* 1989; Stamler *et al* 1994]. In addition, nitric oxide release can be stimulated by a range of agonists including thrombin, bradykinin, substance P and acetylcholine, as well as by shear and mechanical stress [Cooke *et al* 1991]. The increase in eNOS activity evoked by shear stress contributes to the phenomenon of flow associated or mediated vasodilatation, an important

autoregulatory mechanism by which blood flow is regulated in response to ischaemia and hyperaemia [Loscalzo & Vita 1994].

Nitric oxide is released into the abluminal side of the endothelial cells where it activates smooth muscle cell guanylate cyclase and increases intracellular cyclic guanosine monophosphate [Arnold *et al* 1977]. This results in a reduction of intracellular calcium and thereby causes smooth muscle relaxation [Collins *et al* 1986]. The same pathway is involved in the mechanism of action of exogenous nitric oxide donors such as sodium nitroprusside and nitroglycerin. Nitric oxide may also diffuse into vascular lumen where it is predominantly taken up into haemoglobin, but also enters into platelets and inhibits platelet adhesion, activation, and aggregation [Mendelsohn *et al* 1990].

Prostacyclin is another major endothelium-derived vasodilator produced from arachidonic acid through the enzyme cyclo-oxygenase [Moncada *et al* 1977]. It is not constitutively present in endothelial cells, but its rapid synthesis and release is stimulated by bradykinin and adenosine nucleotides that activate adenylate cyclase and cause an increase in intracellular cyclic AMP. Prostacyclin also has anti-thrombotic and anti-platelet activity [Moncada 1982].

In addition, stimulation of the normal endothelium by acetylcholine produces hyperpolarisation of the underlying smooth muscle by an endothelium-derived hyperpolarising factor, which may exert its vascular effects by activating ATP-sensitive potassium channels or smooth muscle sodium-potassium ATPase, although

the precise identity and physiologic role of this factor remains uncertain [Feletou & Vanhoutte 1988].

1.1.4 Endothelium-dependent Vasoconstrictors

Endothelial cells also produce endothelin-1 (ET-1), the most potent vasoconstrictor identified to date [Levin 1995]. ET-1 is not stored in granules, but is formed after transcription of the gene encoding its precursor, preproendothelin-1 following stimulation by hypoxia, shear stress and ischaemia [Boulanger & Luscher 1990]. After removal of a short secretory sequence, preproendothelin-1 undergoes cleavage by an endoprotease to generate big ET-1, which is subsequently converted by endothelin converting enzyme to the biologically active ET-1. ET-1 has a short half-life, and when released from the endothelium, binds to the G-protein coupled ET-A receptor on vascular smooth muscle cell, leading to an increase in intracellular calcium concentration and enhanced vascular tone [Simonson & Dunn 1990]. ET-1 also enhances the vasoconstrictor actions of catecholamines, and vice versa. ET-1 is present in healthy subjects in low concentrations, and contributes to the maintenance of basal vascular resistance [Davenport *et al* 1990].

Platelet activating factor is a phospholipid that is not constitutively present in resting human endothelial cells, but its production can be induced rapidly by humoral and mechanical stimuli including thrombin, angiotensin II, vasopressin and bradykinin. Platelet activating factor acts as a potent vasoconstrictor at high doses in the coronary

circulation in a paracrine fashion, but may also have vasodilator properties at low doses [Cincs *et al* 1998, Montrucchio *et al* 2000].

Angiotensin II, the central product of the renin-angiotensin-aldosterone system, is well known to increase systemic blood pressure via its constrictor effects. The vascular actions of angiotensin II are primarily mediated through the angiotensin II type 1 receptor, a G-protein-coupled receptor expressed on endothelial cells, monocytes and vascular smooth muscle cells. Angiotensin II type 1 receptor activation induces a cascade of cellular responses, including rapid activation of phospholipase C and intracellular calcium leading to enhanced vascular tone. More sustained effects of angiotensin II are mediated by changes in gene transcription. There is also emerging evidence that angiotensin II may play an important role in the pathophysiology of cardiac hypertrophy and remodelling, heart failure, vascular thickening, atherosclerosis and glomerulosclerosis [Kim & Iwao 2000, Brasier *et al* 2002].

1.2 The Endogenous Fibrinolytic System

The endogenous fibrinolytic system consists of a highly regulated enzymatic cascade for extracellular proteolysis [Lijnen & Collen 1995]. Its principal role is to protect the circulation from intravascular fibrin formation and thrombosis that would otherwise result in vessel occlusion and tissue ischaemia. Plasmin, the central enzyme in this system, is formed from the inactive precursor protein, plasminogen,

by the action of plasminogen activators. In the circulation, plasmin degrades the fibrin of haemostatic plugs and thrombi into soluble fibrin degradation products. Inhibition of fibrinolysis occurs at two principal levels: plasminogen activation and plasmin inhibition, through the actions of plasminogen activator inhibitors and α_2–antiplasmin. The physiological importance of the fibrinolytic system is demonstrated by the clinical association between excessive or impaired fibrinolysis with the tendency towards bleeding or thrombosis respectively [Fay *et al* 1992; Booth *et al* 1983; Kohler & Grant 2000]. Recent studies have also suggested that it may contribute to the pathogenesis of atherosclerosis, restenosis, wound healing and malignancy [Danø *et al* 1985; Kohler & Grant 2000]. The plasmin/plasminogen system is also involved in other processes such as cell migration [Saksela & Rifkin 1988], tissue remodelling, ovulation [Strickland & Beers 1976] and angiogenesis.

1.2.1 Initiation of Fibrinolysis

1.2.1.1 Plasminogen and Plasmin

Plasminogen is a 92 kDA single-chain glycoprotein synthesised predominantly by the liver and is present in plasma at a concentration of 1.5 – 2 μM. Plasminogen consists of 791 amino acid residues and contains five homologous disulphide-bonded triple-loop structures known as 'kringles', each composed of 80 – 85 amino acids [Forsgren *et al* 1987]. These kringle domains are crucial in fibrinolysis as they contain binding sites that mediate the specific interactions between plasminogen and fibrin, and between plasmin and α_2–antiplasmin [Collen 1980]. The plasminogen

gene is located on the long arm of chromosome 6 at band q26 or q27 and contains 19 exons covering 52.5 kb of DNA [Pctersen *et al* 1990].

Plasmin is formed from plasminogen as a result of enzymatic cleavage of the Arg 561 – Val 562 peptide bond by plasminogen activators. The mature plasmin molecule is a two-chain trypsin-like serine protease composed of a heavy chain containing the five kringles and a light chain with an active site that is able to degrade fibrin.

1.2.1.2 Plasminogen Activators

There are two main physiological plasminogen activators: tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Whilst t-PA is primarily involved in fibrin dissolution in the circulation, u-PA may contribute to pericellular proteolysis via degradation of matrix components or via activation of latent proteases and growth factors. However, unlike t-PA, u-PA appears not to be produced by most quiescent endothelial cells, and is only expressed by endothelial cells actively involved in wound repair or angiogenesis.

1.2.2 Inhibition of Fibrinolysis

Plasmin and the plasminogen activators are both regulated by serine protease inhibitors (serpins), which contain reactive centres mimicking the natural substrate of each protease.

1.2.2.1 α_2–antiplasmin

Human α_2-antiplasmin is a 67 kDa glycoprotein containing 464 amino acids and is the major physiological inhibitor of plasmin [Holmes *et al* 1987]. Present in the circulation at a relatively high concentration of ≈1 μM, α_2-antiplasmin also has a long plasma half-life and thus a large capacity to neutralise plasmin. The reactive site of α_2-antiplasmin is the Arg 364 – Met 365 peptide bond and its gene is located on chromosome 18, bands p11.1 – q11.2, contains 10 exons and covers approximately 17 kb of DNA.

Although free plasmin is readily inhibited by α_2-antiplasmin, plasmin formed on a fibrin surface is protected from the actions of its inhibitors as both its active and binding sites interact with fibrin. However, in the presence of activated coagulation factor XIII, α_2-antiplasmin can cross-link to polymerising fibrin and inhibits fibrinolysis by two consecutive reactions. It first forms a 1:1 molar reversible complex with plasmin, followed by covalent binding between the carboxyl group of its reactive site and the hydroxyl group of the active site of plasmin [Collen & Linjnen 1991].

1.2.2.2 Plasminogen Activator Inhibitors

Plasminogen activator inhibitor type 1 (PAI-1) is the principal inhibitor of plasminogen activation in man but several other inhibitors also exist. Plasminogen activator inhibitor type 2 (PAI-2) predominantly inhibits u-PA and, to a lesser extent,

t-PA. Not normally present in plasma except during pregnancy [Kruithof *et al* 1987], PAI-2 probably exerts a local rather than systemic role. Plasminogen activator inhibitor type 3 (also known as the protein C inhibitor) has a much lower affinity for u-PA and t-PA than PAI-1 but it is present in plasma at much higher concentrations [Suzuki *et al* 1983]. C1 esterase inhibitor, α_2-macroglobulin and α_1-antitrypsin are broad spectrum inhibitors that have some reactivity towards plasminogen activators [Bennett *et al* 1990] and may play a role when PAI-1 is completely consumed such as during systemic thrombolytic therapy.

1.3 Plasminogen Activators and Inhibitors

1.3.1 Tissue Plasminogen Activator

1.3.1.1 Structure and Function

Tissue-type plasminogen activator is a 70 kDa glycoprotein consisting of 527 amino acid residues, and has a molecular mass of 59 kDa excluding carbohydrates (or 70 kDa including carbohydrates) [Lijnen & Collen 1995]. The protein structure of t-PA contains several autonomous domains: finger-like, growth factor-like, two kringle structures, and the protease domains. The native t-PA protein is a single-chain molecule that is converted into a two-chain form (A and B) by plasmin or other proteases following cleavage of the Arg 275-Ile 276 peptide bond. The two chains are connected by a disulphide bridge, and the resultant molecule is ellipsoidal and

compact, stabilised by strong interactions between the protease, finger and growth factor domains. The A-chain is required for the stimulation of t-PA by fibrin, and B-chain contains the active sites for the activation of plasminogen.

Tissue-type plasminogen activator, a trypsin-like serine protease, is the key enzyme that converts plasminogen to plasmin, and cleaves peptide bonds with arginine or lysine. Although inefficient in activating plasminogen in solution, t-PA-induced fibrinolytic activity is augmented 400-fold in the presence of fibrin [Collen 1980]. Fibrin potentiates plasminogen activation through the binding of both t-PA and plasminogen to the fibrin clot to form a cyclic ternary complex, and thus act as stimulator of plasminogen activation as well as final substrate of generated plasmin.

1.3.1.2 Synthesis and Secretion

Tissue-type plasminogen activator is synthesised mainly in vascular endothelial cells, stored in small dense vesicles and can be released by constitutive or facultative pathways [van den Eijnden-Schrauwen *et al* 1995; Emeis *et al* 1997]. In this latter pathway, t-PA is released following endothelial cell activation through the translocation of a dynamic storage pool rather than increased *de novo* synthesis. The acute release of t-PA can be induced by agents that include thrombin, histamine, bradykinin, substance P, and by sympathetic activation such as exercise and mental stress [Tranquille & Emeis 1989; Newby *et al* 1997a; Brown *et al* 1999].

The gene for t-PA is located on chromosome 8, covers 32.7 kb of DNA, and consists of 14 exons. An enhancer-like regulatory sequence is located more than 2 kb upstream from the transcription initiation site and may be responsible for t-PA expression. Tissue-type plasminogen activator has a plasma concentration of about 5 – 10 ng/ml that varies strongly under different physiological and pathological conditions, and has a short half-life of about 5 minutes due to rapid hepatic clearance [Chandler *et al* 1997].

1.3.1.3 Receptors

There are two major classes of t-PA receptors, responsible for t-PA clearance and for t-PA localisation on cell surfaces. Clearance receptors are associated with endocytosis and lysosomal degradation of t-PA and serve as negative regulators of vascular fibrinolysis. The liver is the main organ responsible for t-PA clearance, and hepatic parenchymal, endothelial and Kupffer cells are all capable of t-PA uptake. These t-PA receptors include a well-characterised mannose receptor present on hepatic endothelium and a low-density lipoprotein receptor related protein identified on parenchymal hepatocytes. Tissue-type plasminogen activator receptors present on vascular endothelial cell surface can promote pericellular plasminogen activation and thereby have profibrinolytic consequences. One such receptor is annexin II which mediates the assembly of plasminogen and t-PA to accelerate and localise plasmin generation [Hajjar *et al* 1994].

1.3.2 Plasminogen Activator Inhibitor Type 1

1.3.2.1 Structure and Function

Plasminogen activator inhibitor-1 is a linear glycoprotein composed of 379 amino acid residues and has a molecular weight of 42 kDa [Lijnen & Collen 1995]. Plasminogen activator inhibitor-1 exists in at least four structurally different forms: active, latent, non-inhibitory substrate and inactive cleaved forms. Synthesised as an active inhibitor, the active PAI-1 is unstable and has a half-life of 30 minutes. It is spontaneously converted into the latent form, which has a half-life of 4 hours in plasma, and can also be partially reactivated by denaturing agents. The non-inhibitory substrate form of PAI-1 does not form stable complexes with t-PA and can be irreversibly degraded by target proteases including t-PA and thrombin. Conversion of the inhibitory PAI-1 into the latent or substrate forms may represent a regulatory mechanism of the fibrinolytic system.

Plasminogen activator inhibitor-1 is the primary inhibitor of plasminogen activation. It first binds rapidly to either t-PA or u-PA, forming a reversible complex with a ratio of 1:1. This is followed by a covalent complex in which the hydroxyl group of the active site of the plasminogen activator forms an ester bond with the carboxyl group of the reactive centre of PAI-1. When this ester bond hydrolyses, the cleaved and inactive derivative of PAI-1 is formed. The t-PA–PAI-1 complex is also eliminated from the circulation by hepatic cells. Vitronectin is a well established co-factor of

PAI-1 that binds and stabilises active PAI-1 in plasma, protecting it against inactivation by oxidants [Reilly *et al* 1994].

1.3.2.2 Synthesis and Secretion

The gene for PAI-1 is located on chromosome 7, covers approximately 12.2 kb DNA and consists of nine exons. Although synthesised by a variety of cell types including endothelial cells, megakaryocytes, monocytes and hepatic cell *in vitro*, the precise source of PAI-1 in plasma is not known. In endothelial cells, the expression of PAI-1 is highly regulated and stimulated by a variety of agonists including endotoxin, interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) and growth factors [Andreasen *et al* 1990].

Plasminogen activator inhibitor-1 has a normal plasma concentration of about 20 ng/ml with platelets representing the main circulating pool. There is an approximately 5-fold molar excess of PAI-1 compared to t-PA, so that most of the t-PA circulates as t-PA–PAI-1 complex.

1.3.3 Diurnal Variation

Both t-PA and PAI-1 show distinct circadian variation [Kluft *et al* 1988]. Plasma concentrations of PAI-1 peak in the morning and reach a trough in the late afternoon and evening. In contrast, t-PA concentrations peak in late afternoon and reach a trough in the morning. The exact reasons for this variation are not known.

1.4 Inflammation

1.4.1 Circulating Inflammatory Cytokines and Markers

1.4.1.1 Tumour Necrosis Factor-α

Tumour necrosis factor-α is a trimeric 17 kDa, 157 amino acid polypeptide produced mainly by monocytes and activated macrophages but also by many other cell types [Azzawi & Hasleton 1999]. It is expressed as a 26 kDa integral transmembrane precursor protein from which the 17 kDa subunit is released after proteolytic cleavage, and exists in both secreted and transmembrane forms. The cytokine is rapidly synthesised and released on demand, and is not stored in the cytoplasm.

Tumour necrosis factor-α is a pleiotropic cytokine and a mediator of systemic responses to injury, sepsis and inflammation [Ferrari 1999]. It activates and suppresses multiple transduction pathways and triggers the release of practically all known mediators of inflammation such as the pro-inflammatory cytokines, IL-1 and interleukin-6 (IL-6), as well as its own production, the final result ranging from haemorrhagic necrosis to extensive fibrosis. However, it also has protective effects and induces oxygen free-radical scavenging enzymes, such as mitochondrial superoxide dismutase, and protective substances such as heat shock proteins [Aggarwal & Natarajan 1996].

Tumour necrosis factor-α acts at the cellular level via both type I (p55) and type II (p75) receptors present on almost all nucleated cell types. Activation by TNF-α of different transduction mechanisms may operate through separate TNF-α receptors, and both receptors can be up or down-regulated under different pathophysiological conditions [Torre-Amione *et al* 1995].

1.4.1.2 Interleukin-6

Interleukin-6 is a 26 kDa protein that consists of 212 amino acids with a hydrophilic signal sequence of 28 residues [Kerr *et al* 2001]. The gene for IL-6 is mapped to chromosome 7p21, consists of five exons covering 5 kb DNA, and has a fairly complex transcriptional regulation involving 3 initiation sites. Interleukin-6 is synthesised by a variety of cell types including activated monocytes and macrophages, endothelial cells, adipose cells, and the Th-2 subset of T-helper cells. Expression of IL-6 is regulated in response to a number of stimuli including endotoxins, IL-1, TNF-α, Interleukin-4 and interferon-γ, although different cell types may respond differently to these stimuli.

Interleukin-6 is a multifunctional cytokine with a broad range of actions and plays a central role in the host defence mechanism. Apart from the modulation of immune responses and induction of acute phase reactant release from hepatocytes during immunologic and inflammatory reactions, IL-6 also participates in many biologic and pathophysiological processes such as bone metabolism, thrombopoiesis,

epidermal proliferation and cancer [Kurihara *et al* 1990; Hill *et al* 1990; Grossman *et al* 1989; Zhang *et al* 1989].

The functional effects of IL-6 are mediated through a specific receptor complex that consists of two membrane-anchored glycoproteins, gp80 and gp130, which are responsible for ligand binding and initiation of the signal transduction cascade respectively.

1.4.1.3 C-reactive Protein

C-reactive protein (CRP) is a major acute-phase plasma protein originally isolated as a protein that binds to the C-polysaccharide of the *Streptococcus pneumoniae* cell wall. It consists of five identical 23 kDa non-covalently associated subunits each made up of 206 amino acids, and arranged in a ring resembling a doughnut. The CRP gene has been mapped to human chromosome 1, and contains 2263 nucleotides with a single intron [Volanakis 2001].

C-reactive protein is synthesised and secreted mainly by the liver and has a plasma half-life of 19 hours. Interleukin-6 is the principal inducer of the CRP gene, while IL-1, glucocorticoids and complement activation products can act synergistically with IL-6 to enhance its effects [Toniatti *et al* 1990]. Under physiologic conditions, CRP is synthesised at low rates, but during an inflammatory process in response to injury or infection, its level can be elevated 100-fold within 24 – 48 hours.

The main biologic function of CRP is to recognise pathogens and damaged cells of the host and to mediate their elimination by recruiting the complement system and phagocytic cells. Thus CRP level may reflect the degree of underlying inflammatory response and provide a useful measure of immune injury to tissues. Elevated in many inflammatory disorders, serum CRP concentrations have been widely used to predict clinical outcomes [Yeh *et al* 2001].

1.4.2 Inflammation and Atherosclerosis

Arterial inflammation has emerged as central to the progression of atherothrombosis, a process initiated through an injury to the vascular endothelium [Ross 1999]. Activation of the vascular endothelium leads to increased expression of leucocyte adhesion proteins, reduced anticoagulant activity and the release of growth factors, inflammatory mediators and cytokines. Continued inflammation results in leucocyte and monocyte recruitment, induction of atheroma formation and further arterial damage. Cycles of further damage cause plaque expansion and disruption that can lead to angina, crescendo angina and acute coronary syndromes including myocardial infarction.

The pro-inflammatory cytokines, IL-6 and TNF-α, have been implicated in the initiation and maintenance of systemic and vascular inflammation associated with atherosclerosis and ischaemic heart disease [Biasucci *et al* 1996; Vaddi *et al* 1994; Ridker *et al* 2000a, 2000b]. Together with IL-1, they are often co-activated and share overlapping biological activities [Pober & Cotran 1990]. In particular, they share the

ability to initiate or suppress gene expression for several proteins and can activate endothelial cells, cause expression of adhesion molecules and promote thrombogenesis. Although usually absent or only present in small quantities in normal cardiac tissue, TNF-α, IL-6 and CRP have all been demonstrated in atherosclerotic lesions [Lagrand *et al* 1999; Reynolds & Vance 1987; Rus *et al* 1996; Barath *et al* 1990]. Furthermore, TNF-α and IL-6 expression increases with the severity of the lesion, suggesting that they may play a role in disease evolution.

1.4.3 Inflammation as Cardiovascular Risk Factor

Epidemiological and observational studies have suggested an association of systemic infection and inflammation with the risk of major adverse cardiovascular events. Cardiovascular death rates are greater in the winter and are increased by epidemics of influenza. Infections by organisms, such as *chlamydia pneumoniae* and *herpes simplex type I*, also seem to be associated with an increased risk [Roivainen *et al* 2000]. Moreover, approximately 4% of bacteraemic patients develop an acute myocardial infarction within a month of infection and up to 10% of all strokes are preceded by a bacterial infection [Valtonen *et al* 1993]. Increased cardiovascular morbidity and mortality is also seen following respiratory tract infections, severe illnesses requiring intensive care and surgery [Meier *et al* 1998; Quartin *et al* 1997; Mamode *et al* 1995]. A recent large study further demonstrated that acute respiratory tract infections as well as urinary tract infections are associated with a transient increase in the risk of myocardial infarction and stroke [Smeeth *et al* 2004].

Plasma concentrations of TNF-α, IL-6 and CRP are elevated in patients with cardiovascular diseases [Liuzzo *et al* 1994; Biasucci *et al* 1996; Ridker *et al* 2000a, 2000b, 2000c]. In previously healthy individuals, elevated plasma CRP and IL-6 concentrations have been shown to predict the development of cardiovascular disease and the risk of a first myocardial or cerebral infarction [Ridker *et al* 1997, 2000b, 2000c]. In addition, elevated plasma IL-6 and TNF-α concentrations predict adverse cardiovascular outcome in patients with acute coronary syndromes [Biasucci *et al* 1999; Ridker *et al* 2000a]. Reflecting its anti-inflammatory action, the beneficial effects of aspirin in reducing cardiovascular risk are proportional to the plasma C-reactive protein concentration [Ridker *et al* 1997].

These data suggest a link between chronic low-grade inflammation and the slow process of atherogenesis, as well as an association between acute systemic inflammation and a transiently increased risk of an acute cardiovascular event. Furthermore, it appears that the effects of inflammation on cardiovascular risk may be generic, rather than linked to specific types of infection. Although inflammation may be argued to be a response to, rather than a cause of atherosclerosis, recent work suggests that these inflammatory markers may also play a direct pathogenic role in atherosclerotic lesion formation, and plaque destabilisation [Ross 1999; Pasceri *et al* 2000].

1.4.4 Inflammation and Fibrinolysis

In vitro studies have demonstrated that inflammatory cytokines can alter the fibrinolytic and haemostatic function of the endothelium [Bevilacqua *et al* 1986; Schleef *et al* 1988]. Recombinant human TNF-α reduces t-PA and stimulates PAI-1 release from cultured human umbilical vein endothelial cells and also exerts a net procoagulant effect by enhancing tissue factor and thrombin-induced von Willebrand factor expression.

Using lipopolysaccharide administration in an *in vivo* model of systemic inflammation, experimental endotoxaemia in chimpanzees induced an initial increase in, followed by a marked suppression of fibrinolytic activity [Biemond *et al* 1995]. This response is markedly inhibited by the co-administration of anti-tumour necrosis factor-α antibodies. These findings have been subsequently confirmed in clinical studies of healthy volunteers where an identical pattern of responses to endotoxaemia and anti-TNF-α antibodies was observed [van der Poll *et al* 1994a, 1997]. Moreover, systemic administration of TNF-α in healthy volunteers causes a rapid fibrinolytic response that precedes a subsequent and more prolonged activation of the coagulation system [van der Poll *et al* 1990, 1991]. Primate and clinical studies of IL-6 show a distinct response from that obtained with TNF-α. Interleukin-6 administration stimulates the coagulation system and augments the coagulation response to endotoxaemia but does not appear to have a distinct action on the fibrinolytic system [van der Poll *et al* 1994b; Stouthard *et al* 1996].

1.5 Endothelial Dysfunction and Cardiovascular Disease

1.5.1 Clinical Assessment of Endothelial Function

In vivo techniques offer distinct advantages over *in vitro* studies in the assessment of vascular endothelial function as the blood vessels are exposed to physiological pressure and local and circulating vasoactive and growth factors [Webb 1995]. In addition, they also have intact nervous mechanisms and have not been exposed to potentially significant vessel trauma or hypoxia. In the last 15 years, several techniques have been developed to assess arterial endothelial function in the coronary and peripheral circulation in health and disease. Most of these have investigated the ability of normal endothelium to release nitric oxide in response to pharmacologic or mechanical stimuli. However, whilst endothelium-dependent vasomotion is important, it may not be representative of other aspects of endothelial function, such as the regulation of haemostasis, fibrinolysis and inflammation. Recently, the *in vivo* assessment of endothelial fibrinolytic function has become possible [Newby *et al* 1997a].

1.5.1.1 Coronary Circulation

In the coronary artery, quantitative angiography can be used to examine the changes in diameter in response to intra-coronary infusions of endothelium-dependent vasodilators such as acetylcholine [Ludmer *et al* 1986]. In healthy arteries, acetylcholine stimulates the endothelial release of nitric oxide resulting in a

vasodilatory response, whereas subjects with endothelial dysfunction may demonstrate impaired acetylcholine mediated vasodilatation or paradoxical vasoconstriction. Endothelial function of the coronary microcirculation can also be assessed with intra-coronary Doppler techniques to measure coronary blood flow [Drexler & Zeiher 1991]. Other non-invasive tests include Doppler echocardiography, positron emission tomography and phase-contrast magnetic resonance imaging.

1.5.1.2 Peripheral Circulation

The use of intra-coronary techniques is however limited by their invasive nature and therefore has restricted application in research. Non-invasive or minimally invasive methods have been developed to determine endothelial function in peripheral arteries as a surrogate for coronary circulation. Two of the commonest modalities adopted are brachial artery ultrasound and forearm venous occlusion plethysmography.

Brachial artery ultrasound is a non-invasive technique that evaluates flow-mediated vasodilatation, an endothelium-dependent function, in the brachial artery. [Celermajer *et al* 1992, Corretti *et al* 2002]. This stimulus provokes the endothelium to release nitric oxide with subsequent vasodilatation that can be imaged and quantified as an index of vasomotor function. In this test, the brachial artery is imaged above or below the antecubital fossa in the longitudinal plane using an ultrasound system equipped with a high-frequency vascular transducer, two-dimensional imaging, and colour and spectral Doppler. Arterial occlusion is

typically created by inflating a sphygmomanometer cuff to suprasystolic pressure placed either above the antecubital fossa or on the forearm for a standardised length of time. This causes ischaemia and consequent dilatation of downstream resistance vessels via autoregulatory mechanisms. Subsequent cuff deflation induces a brief high-flow state through the brachial artery (reactive hyperaemia) to accommodate the dilated resistance vessels. The resulting increase in shear stress causes the brachial artery to dilate. Flow-mediated vasodilatation can then be quantified as percentage change in post-stimulus brachial artery diameter compared to baseline, or in terms of absolute change in diameter. In most studies to date, an exogenous nitric oxide donor, such as sublingual nitroglycerine has also been given to determine the maximum obtainable vasodilator response, and to serve as a measure of endothelium-independent vasodilatation reflecting vascular smooth muscle function.

However, despite its widespread use, there are technical and interpretive limitations to the use of brachial artery ultrasound [Corretti *et al* 2002]. Furthermore, there are distinct phenotypic differences between conduit artery and microvascular vessel endothelial cells, the major determinants of peripheral resistance. Therefore assessment of flow mediated vasodilatation may not be wholly indicative of tissue perfusion. Quantification of the endothelial fibrinolytic function is also not possible with this technique.

In contrast, the use of bilateral forearm venous occlusion plethysmography combined with unilateral brachial artery infusion of vasoactive drugs and venous sampling provides a powerful and reproducible tool of direct assessment of vascular vasomotor

and fibrinolytic responses in the forearm resistance vessels [Newby *et al* 1997a, 1997b, 1998a]. This technique determines the change in forearm blood flow in response to intra-arterial administration of endothelium-dependent agonists such as acetylcholine, substance P and bradykinin, and the endothelium-independent vasodilators such as sodium nitroprusside. In addition, simultaneous venous sampling for t-PA and PAI-1 measurements allows the assessment of the acute endothelial fibrinolytic capacity of the forearm circulation.

Although these peripheral artery techniques provide only surrogate measures of the coronary endothelial function, several reports have indicated that they correlate with findings from intra-coronary studies [Anderson *et al* 1995; Newby *et al* 1999, 2001], and have provided important insights into the pathophysiology of atherogenesis and the influence of cardiovascular risk factors.

1.5.2 Endothelial Dysfunction and Atherosclerosis

Endothelial dysfunction or injury is widely regarded as an important early event in atherosclerosis. There is now extensive evidence of endothelial dysfunction and abnormal endothelium-dependent vasomotion in patients with atherosclerosis and its associated risk factors, such as smoking, hypercholesterolaemia, diabetes mellitus, aging and hyperhomocysteinaemia [Celermajer *et al* 1993a, 1993b; Zeiher *et al* 1993; Calver *et al* 1992; Chowienczyk *et al* 1992]. Moreover, recent studies have shown that endothelium-dependent vasodilatation is an independent predictor of adverse cardiovascular events [Schachinger *et al* 2000; Halcox *et al* 2002].

Impaired endothelial function actively participates in the process of lesion formation by promoting the early and late mechanisms of atherosclerosis that include upregulation of adhesion molecules, increased chemokine secretion and leukocyte adherence, increased cell permeability, enhanced low-density lipoprotein oxidation, platelet activation, cytokine elaboration and vascular smooth muscle cell proliferation and migration [Celermajer 1997; Drexler 1997; Ross 1999]. Furthermore, endothelial dysfunction can modulate the clinical course of atherosclerosis by altering plaque architecture, vulnerability of the lesion and the likelihood of rupture, and thus accelerate the development of unstable coronary syndromes.

1.5.3 Endothelial Dysfunction and Endogenous Fibrinolysis

Endogenous fibrinolysis plays an important role in the spontaneous reperfusion of infarct-related arteries after acute myocardial infarction and can occur in as many as 30% of patients within 12 hours [Armstrong *et al* 1989; De Wood *et al* 1980; Rentrop *et al* 1989]. The rapid mobilisation of t-PA from the endothelium is crucial, as thrombus dissolution is much more effective if t-PA is incorporated during, rather than after thrombus formation [Fox *et al* 1984]. Although it would be anticipated that high plasma t-PA concentrations should protect against subsequent coronary events, epidemiological studies of healthy men and patients with ischaemic heart disease have indicated that higher plasma t-PA antigen concentrations positively predict future coronary events [Thompson *et al* 1995; Ridker *et al* 1993]. This is explained

by the concomitant elevation of PAI-1 that forms a complex with t-PA and thereby causes an overall reduction in free t-PA activity [Meade *et al* 1993]. However, basal concentrations of fibrinolytic factors may not reflect the capacity of endothelial cells to release t-PA from intracellular storage pools.

Assessment of this acute endogenous fibrinolytic capacity has been recently described [Newby *et al* 1997a]. Studies have shown that the acute release of t-PA is reduced following experimental 'endothelial dysfunction' with nitric oxide synthase inhibitor, as well as in smokers with and without coronary artery disease when compared to non-smokers [Newby *et al* 1998b, 1999, 2001]. However, the association between the endogenous fibrinolytic capacity and inflammation and other cardiovascular risk factors such as hyperhomocysteinaemia has not been established.

1.5.4 Endothelial Dysfunction and Hyperhomocysteinaemia

Several prospective and case-control studies have shown that elevated plasma homocysteine concentrations are an independent risk factor for the development of atherothrombotic vascular disease as well as a prognostic marker in ischaemic heart disease [Clarke *et al* 1991; Stampfer *et al* 1992; Welch & Loscalzo 1998]. Although the mechanism of vascular damage is unclear, homocysteine may promote atherogenesis through oxidative endothelial injury that is mediated by cytotoxic reactive oxygen species [Stamler *et al* 1993; Lentz *et al* 1996; Loscalzo 1996; Kanani *et al* 1999]. Indeed, acute and chronic hyperhomocysteinaemia are associated

with impaired vasomotor tone [Celermajer *et al* 1993a; Tawakol *et al* 1997; Kanani *et al* 1999].

Hyperhomocysteinaemia is a prothrombotic state [Welch & Loscalzo 1998]. Previous studies have shown that hyperhomocysteinaemia induced by oral methionine loading is associated with alterations in endogenous fibrinolysis in healthy subjects and patients with premature vascular disease [Labinjoh *et al* 2001; Freyburger *et al* 1997]. However, the influence of chronic hyperhomocysteinaemia on the acute fibrinolytic capacity is unknown and is the subject of debate [Kuller & Evans 1998]. Vitamin supplementation with folic acid, vitamin B_6 and vitamin B_{12}, may reduce plasma homocysteine concentrations [Homocysteine Lowering Trialists' Collaboration 1998]. Whilst endothelial function is enhanced following treatment with folic acid in patients with hyperhomocysteinaemia [Woo *et al* 1999, 2002], the response in patients with coronary artery disease has been variable [Chambers *et al* 2000; Title *et al* 2000; Thambyrajah *et al* 2001; van Dijk *et al* 2001; Doshi *et al* 2002]. Furthermore, the association between elevated plasma homocysteine concentrations and resistance vessel vasomotor responses in patients with established coronary artery disease has not been characterised.

1.5.5 Endothelial Dysfunction and Coronary Stent Thrombosis and In-stent Restenosis

Coronary artery stent implantation is a valuable adjunct to percutaneous transluminal coronary angioplasty. However, there is a small but significant risk of acute coronary stent thrombosis [Cutlip *et al* 2001] and in-stent restenosis that can have devastating consequences including myocardial infarction and death [Albiero *et al* 1997]. Whilst procedure-related complications such as persistent dissection, longer stent length and final minimal lumen diameter may be implicated in some cases of stent thrombosis or restenosis [Cutlip *et al* 2001], no underlying precipitant can be identified in many patients.

Stent thrombus formation is principally initiated by platelet aggregation which, in the absence of effective endothelium-derived vasoregulation and fibrinolysis, is then stabilised by the deposition of a fibrin mesh. The chronology of in-stent restenosis has been described as early thrombosis, followed by thrombus endothelialisation and infiltration by lymphocytes and monocytes, and finally smooth muscle cell migration and proliferation within the resolving thrombus [Schwartz *et al* 1993; Miller *et al* 1996; Komatsu *et al* 1998]. The initiation, propagation and stabilisation of acute stent thrombosis and in-stent restenosis are therefore dependent on several components: platelet aggregation, endothelial function, coagulation and fibrinolysis.

1.6 Hypothesis and Aims

Although recent studies have investigated the effects of experimental inflammation on the venous circulation and arterial endothelial function [Bhagat & Vallance 1997; Hingorani *et al* 2000], the direct influence of inflammatory cytokines on arterial endothelial function has not been explored. Furthermore, little is known about the effects of systemic or local vascular inflammation on the acute fibrinolytic capacity. The potential role of endogenous fibrinolysis and endothelial dysfunction in the pathogenesis of prothrombotic conditions such as hyperhomocysteinaemia and coronary stent thrombosis or in-stent restenosis have also not been investigated.

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

1. Acute systemic inflammation impairs endothelial function and alters the acute endothelial fibrinolytic capacity
2. Direct intra-arterial TNF-α, IL-6 and endotoxin stimulates endogenous fibrinolysis in the forearm vascular bed
3. Direct intra-arterial cytokines impair endothelium-dependent vasodilatation and the acute release of t-PA
4. Endothelium-dependent vasomotion and fibrinolysis are impaired in patients with recent myocardial infarction and hyperhomocysteinaemia
5. Vitamin supplementation would improve endothelial function in patients with recent myocardial infarction and hyperhomocysteinaemia

6. Endothelium-dependent vasomotion and endogenous fibrinolysis is impaired in patients with acute stent thrombosis or in-stent restenosis

The aims of this thesis were

In healthy male volunteers (Chapter 3),

- To determine whether a mild acute systemic inflammatory response induced by *Salmonella typhi* vaccination impairs endothelium-dependent vasodilatation
- To ascertain if the acute release of t-PA in the forearm circulation is altered by acute systemic inflammation

In healthy male volunteers (Chapter 4),

- To establish an *in vivo* model of local vascular inflammation
- To examine the direct effects of local intra-arterial TNF-α, IL-6 and endotoxin on basal vascular tone and the endogenous release of the fibrinolytic and coagulant factors, t-PA, PAI-1 and von Willebrand factor
- To assess the effects of intra-arterial TNF-α infusion on endothelium-dependent and -independent vasodilatation
- To ascertain if TNF-α impairs the acute fibrinolytic capacity

In patients with recent myocardial infarction (Chapter 5),

- To determine if patients with co-existing hyperhomocysteinaemia have impaired endothelium-dependent vasodilatory and fibrinolytic responses compared to those with normal plasma homocysteine concentrations
- To confirm that vitamin supplementation with folic acid, vitamins B6 and B12, lowers plasma homocysteine concentration
- To assess the effects of vitamin supplementation on endothelial vasomotor and fibrinolytic responses in those with hyperhomocysteinaemia

In patients with previous acute coronary stent thrombosis or in-stent restenosis (Chapter 6),

- To determine if forearm endothelium-dependent vasodilatation is impaired
- To assess whether platelet aggregation is abnormal
- To examine if the basal and acute fibrinolytic function is altered

Chapter 2

Methods:

Assessment of Endothelial Vasomotor and Fibrinolytic Function

2.1 Subjects

2.1.1 Subject Recruitment

Healthy subjects were recruited from medical students and a database of healthy volunteers. Patient subjects were recruited from the Cardiology Out-Patient Clinics or the Cardiology wards of the Royal Infirmary of Edinburgh, The Lothian University Hospitals NHS Trust.

2.1.2 Subject Preparation

None of the normal healthy volunteers received vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study. Medications were withheld on the day of the study in all patient subjects. All subjects abstained from alcohol and caffeine-containing drinks for 24 hours and from food and tobacco for at least four hours before each study. All studies were conducted in a quiet, temperature-controlled room maintained at 22 – 25°C.

2.1.3 Ethics Committee Approval and Consent

All studies were undertaken with the approval of the Lothian Research Ethics Committee and in accordance with the Declaration of Helsinki (1996) of the World Medical Association. The written informed consent of each subject or patient was obtained before entry into the study.

2.2 Drugs

2.2.1 Endothelium-dependent and –independent Agonists

Pharmaceutical-grade compounds were used for all intra-arterial infusions of vasoactive agents. The endothelium-dependent vasodilators, acetylcholine (Cibavision Ophthalmics, Southampton, United Kingdom), substance P and bradykinin (both from Clinalfa, Läufelfingen, Switzerland), and the endothelium-independent vasodilator, sodium nitroprusside (David Bull Laboratories, Faulding, United Kingdom) were administered following dissolution in 0.9% normal saline (Baxter Healthcare Ltd., Berkshire, United Kingdom). Substance P and bradykinin both induce endothelium-dependent vasodilatation and stimulate the acute release of tissue-type plasminogen activator (t-PA) from the forearm vascular bed. Neither sodium nitroprusside nor acetylcholine affects plasma t-PA or plasminogen activator inhibitor type-1 (PAI-1) concentrations in the forearm [Newby *et al* 1997a; Brown *et al* 1999]. All solutions were freshly prepared on the day of study.

2.2.2 Inflammatory Stimulus

A systemic inflammatory response was generated with typhoid vaccination using *Salmonella typhi* capsular polysaccharide vaccine 0.025 mg (Typhim Vi, Aventis Pasteur MSD, Berkshire, United Kingdom) [Hingorani *et al* 2000].

Lipopolysaccharide (lot G-1, USPCI, Rockville, Maryland, USA) [Pernerstorfer *et al* 1999] and the pro-inflammatory cytokines, tumour necrosis factor-α (TNF-α, Knoll

Pharmaceuticals, Germany) [Janik *et al* 1999] and interleukin-6 (IL-6, Novartis Pharma AG, Basel, Switzerland) [Tsigos *et al* 1997], were administered following dissolution in 0.9% saline in order to generate a direct local inflammatory response. Lipopolysaccharide, TNF-α and IL-6 were prepared as stock solutions and stored at -80 °C in aliquots.

2.2.3 Vitamin Supplementation

Four weeks of sucrose placebo tablets (Thornton & Ross Ltd, Huddersfield, United Kingdom) or vitamin supplementation was given as a once daily oral preparation. Vitamin supplementation was administered as a combination therapy of folic acid (5 mg; Alpharma, Barnstaple, United Kingdom), vitamin B_6 (pyridoxine 10 mg; B R Pharma Ltd., Barnet, Untied Kingdom) and vitamin B_{12} (cyanocobalamin 100 μg; Goldshield Pharmaceuticals Ltd., Croydon, Untied Kingdom). Vitamin supplementation at these doses has been shown to be safe and effective and may reduce plasma homocysteine concentrations [Homocysteine Trialists' Collaboration 1998].

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-standard wire gauge steel needle (Cooper's Needle Works Ltd., Birmingham, United Kingdom) under 1% lidocaine (Xylocaine; Astra Pharmaceuticals Ltd., Kings Langley, United Kingdom) local anaesthesia and attached to a 16-gauge epidural catheter (Portex

Ltd., Hythe, United Kingdom) (Figure 2.1). Patency was maintained by infusion of saline (0.9% sodium chloride) via a MS2000 syringe infusion pump (Graseby Medical, Watford, United Kingdom). The total rate of intra-arterial infusion was maintained constant throughout all studies at 1 mL/min.

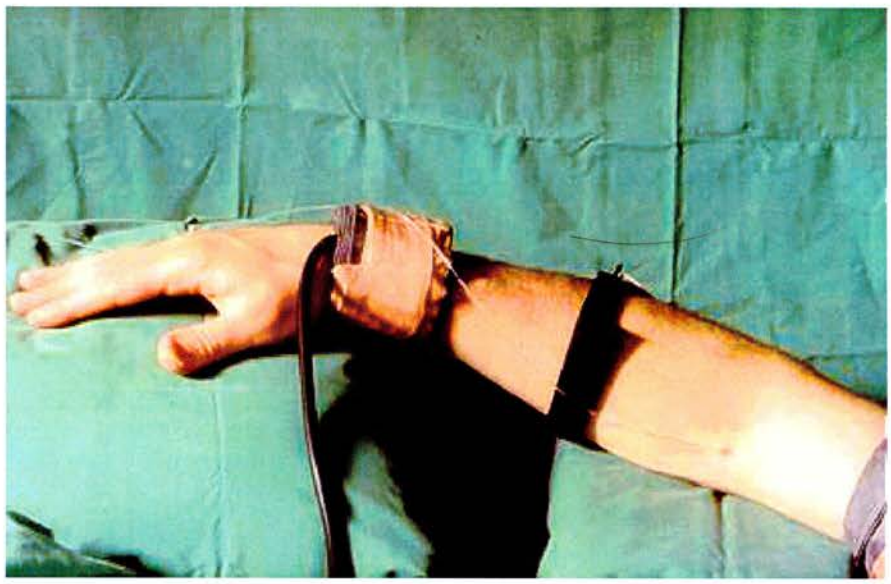

Figure 2.1 Brachial artery cannulation.

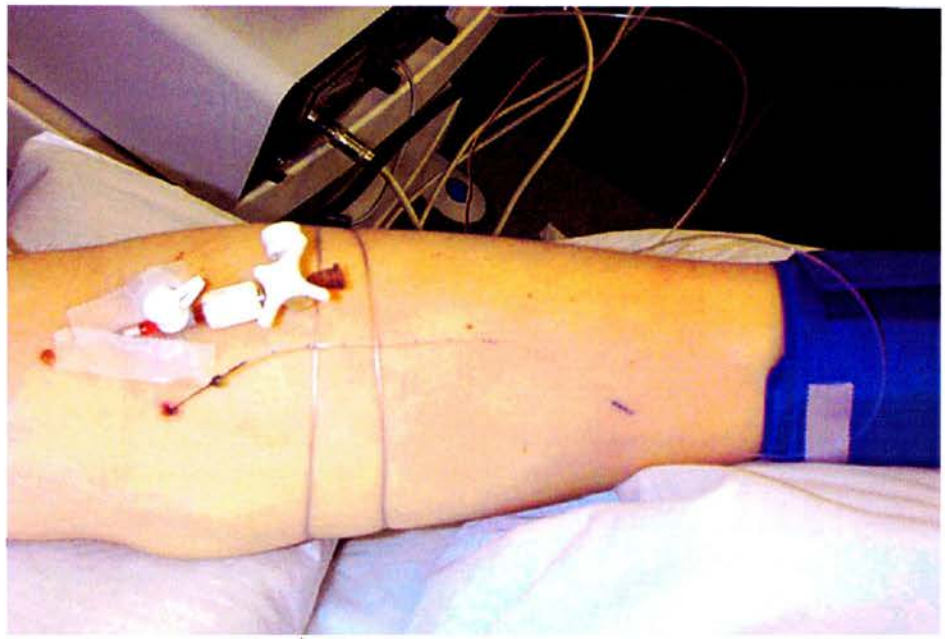

Figure 2.2 Application to the forearm of the mercury-in-silastic strain gauge, brachial artery and venous cannulae, and wrist cuffs.

2.3.2 Forearm Blood Flow

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 (Figures 2.2 and 2.3) [Webb 1995]. During measurement periods the hands were excluded from the circulation by rapid inflation of the wrist cuffs to 220 mmHg using E20 rapid cuff inflators (DE Hokanson® Inc, Bellevue, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mmHg for 8 sec in every 10 sec to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 Strain Gauge Plethysmograph (DE Hokanson®) was processed by a Powerlab®/400 analogue-to-digital converter and Chart™ v3.4.6 software (AD Instruments Ltd., Castle Hill, Australia) and recorded onto an Acer 513TE computer

(Acer Incorporated, Taipei, Taiwan). Calibration was achieved using the internal standard of the plethysmograph.

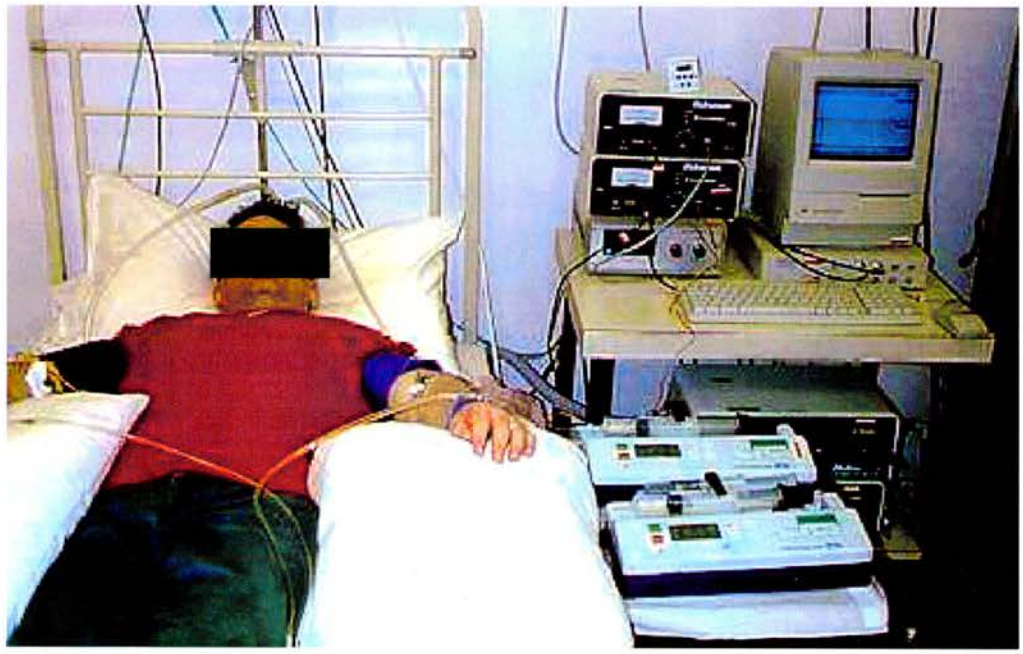

Figure 2.3 Forearm venous occlusion plethysmography.

2.3.3 Blood Pressure and Heart Rate

Blood pressure and heart rate was monitored in the non-infused arm at intervals throughout each study with a semiautomated noninvasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan). Blood pressure was measured immediately after forearm blood flow recordings to avoid any effects of the venous congestion caused by this procedure on these measurements.

2.4 Analysis of Blood Samples

2.4.1 Venous Sampling

Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms (Figure 2.2). Fasting venous blood was withdrawn into lithium heparin, ethylene diamine tetraacetic acid and serum bead tubes (Monovette®, Sarstedt, Nümbrecht, Germany) for IL-6, C-reactive protein (CRP) and homocysteine assays respectively. Before and during intra-arterial drug infusion, venous blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool® Stabilyte™, Umeå, Sweden; for t-PA assays) and trisodium citrate tubes (Monovette®; for PAI-1 assays). All samples were kept on ice before being centrifuged at 2000*g* for 30 min at 4°C except ethylene diamine tetraacetic acid samples that were centrifuged at 1000*g* for 10 min at 20°C. Platelet-free plasma was decanted and stored at –80°C before assay.

2.4.2 Laboratory Assays

2.4.2.1 Fibrinolytic and Haemostatic Assays

Plasma t-PA and PAI-1 antigens concentrations were determined using enzyme-linked immunosorbent assays (Coaliza® t-PA and Coaliza® PAI-1 respectively, Chromogenix AB, Mölndal, Sweden) [Newby *et al* 1997a]. Plasma t-PA and PAI-1 activities were determined by a photometric method (Coatest® t-PA and Coatest® PAI-1, Chomogenix 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 sensitivitics of the assays were 0.5 ng/mL, 2.5 ng/mL, 0.10 IU/mL and 5 AU /mL respectively.

Von Willebrand factor antigen was determined 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.

2.4.2.2 Inflammatory Parameters

Plasma TNF-α, IL-6 and CRP concentrations were determined using specific enzyme-linked immunosorbent assays (Quantikine human TNF and IL-6 immunoassays, R&D Systems, Abingdon, Untied Kingdom; CRP ELISA, Eurogenetics, Belgium respectively). White cell count was determined using an automated Coulter counter (Beckman-Coulter ACt.8, High Wycombe, Untied Kingdom). Intra-assay coefficients of variation were 4.2% and 5.2% for IL-6 and TNF-α antigen, and inter-assay coefficients of variability were 6.4% and 7.4% respectively. The sensitivities of the assays were 0.7 pg/mL and 0.1 pg/mL respectively.

2.4.2.3 Homocysteine and Vitamin Assays

Plasma homocysteine and serum folate and vitamin B_{12} concentrations were determined using enzyme immunoassay (Axis® Homocysteine EIA, Axis-Shield

AS, Oslo, Norway; Bayer Immuno 1® automated immunoassay analyser, Bayer AG, Leverkusen, Germany respectively).

2.4.2.4 Haematocrit Measurement

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

2.4.2.5 Thrombophilia Screen

Venous blood was screened for anti-thrombin, protein S and protein C deficiencies, factor V (Leiden) and prothrombin A20210G genotypes and anti-phospholipid antibodies.

2.4.3 Platelet Aggregometry

Fasting venous blood (30 mL) was collected into trisodium citrate tubes (Monovette®) and immediately centrifuged at 120*g* for 10 min to obtain platelet-rich plasma, which was aspirated, adjusted to give a platelet count of 250 x 10^9 /L and pre-warmed to 37°C. Aggregation studies were performed on the platelet rich plasma 30-40 min after blood sampling using a standard optical technique (Chronolog Ca560 aggregometer; Labmedics, Stockport, United Kingdom) [Labinjoh *et al* 2001] with the following agonists: adenosine diphosphate (ADP, 0.5 – 10 μmol/L), the thromboxane A2 analogue U46619 (0.5 – 6 μmol/L), thrombin (200 –1000 mU/L) and collagen (1 – 5 μg/mL). Responses were recorded 5 min after addition of agonists and the peak response adjusted for platelet count (250 x 10^9/L).

Platelet count was measured using an automated Coulter counter (Act.8 Coulter Counter; Beckman-Coulter).

2.5 Data Analysis and Statistics

2.5.1 Forearm Blood Flow

Plethysmographic data were extracted from the Chart™ data files and forearm blood flow was calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel 97, Microsoft Corporation, Cambridge, USA) (Figure 2.4). Recordings for the first sixty seconds after wrist cuff inflation were not used because of the variability in blood flow and reflex vasoconstriction this causes [Kerslake DMcK 1949]. The last five linear plethysmographic recordings in each 3 min measurement period were calculated and averaged for each arm.

2.5.2 Fibrinolytic Activity

The estimated net release of t-PA antigen and activity were defined as the product of the infused forearm plasma flow (based on the mean haematocrit, Hct, and the infused forearm blood flow, FBF) and the concentration difference between the infused ($[\text{t-PA}]_{inf}$) and noninfused ($[\text{t-PA}]_{Noninf}$) arms [Newby *et al* 1997a] using the formula,

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

The area under the curve (AUC) was calculated for estimated net t-PA antigen and activity release during bradykinin and substance P infusion. Because basal t-PA concentrations were altered by pre-treatment with TNF-α, t-PA antigen and activity release during bradykinin infusion was calculated by subtracting the mean t-PA release before, and 15 min after cessation of bradykinin infusion (Chapter 4).

2.5.3 General

Data were examined, where appropriate, by analysis of variance (ANOVA) with repeated measures and two-tailed paired Student's *t*-test using Statview (SAS Institute, Inc). All results are expressed as mean±SEM. Statistical significance was assigned at the 5% level.

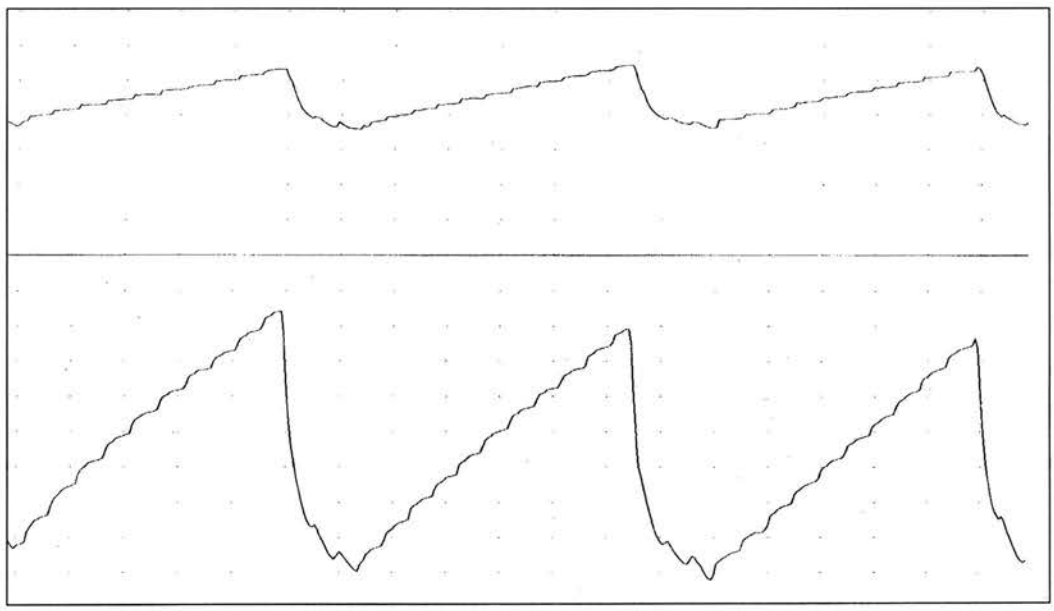

Figure 2.4 Plethysmographic graphs of blood flow from the infused forearm (administered with intra-arterial vasodilators) (lower panel) compared with the non-infused (control) forearm (upper panel).

Chapter 3

The Effects of Acute Systemic Inflammation on Endothelium-Dependent Vasodilatation and Tissue Plasminogen Activator Release in Men

Chia S, Ludlam CA, Fox KAA, Newby DE.

Acute Systemic Inflammation Enhances Endothelium-Dependent Tissue Plasminogen Activator Release in Men.

J Am Coll Cardiol. 2003;**41**:333-9.

3.1 Summary

The current study was designed to investigate the effects of acute systemic inflammation on the endogenous fibrinolytic capacity *in vivo* in man. Systemic inflammation and endogenous fibrinolysis play a major role in the pathogenesis of coronary artery disease. Although previous studies have shown impaired endothelium-dependent vasomotor function, the effects of inflammation on the endothelial release of the fibrinolytic factor, tissue plasminogen activator (t-PA) are unknown. In a double-blind randomised placebo-controlled crossover trial, we administered a mild inflammatory stimulus, *Salmonella typhi* vaccine, or saline placebo to eight healthy men on two separate occasions. Six hours after vaccination, blood flow and plasma fibrinolytic variables were measured in both arms during intra-brachial infusions of bradykinin (40–1000 pmol/min), acetylcholine (5–20 μg/min), and sodium nitroprusside (2–8 μg/min). Compared to placebo, *S. typhi* vaccination caused a rise in white cell count (11.1 vs 7.9 $x10^9$/L; P=0.004) and plasma interleukin-6 concentrations (6.9±1.4 vs 1.6±0.4 pg/mL; P=0.01), and a significant augmentation of t-PA antigen (45±9 vs 24±8 ng/100 mL/min at peak dose; P=0.016) and activity (104±15 vs 54±12 IU/100 mL/min; P=0.006) release during bradykinin infusion. Forearm blood flow increased in a dose-dependent manner following bradykinin, acetylcholine and sodium nitroprusside infusions (P<0.001) but this was unaffected by vaccination. Acute systemic inflammation augments local forearm t-PA release in man. This suggests that acute inflammation may invoke a protective response by enhancing the acute endogenous fibrinolytic capacity in healthy man.

3.2 Introduction

Atherosclerosis is widely recognised to be an inflammatory disease process involving dysfunction of the vascular endothelium [Ross 1999]. This dysfunction leads to increased expression of leucocyte adhesion molecules, reduced anticoagulant activity and the release of growth factors, inflammatory mediators and cytokines. Continued inflammation leads to leucocyte and monocyte recruitment, arterial damage and atherogenesis. Further cycles of damage cause plaque expansion and disruption that may lead to angina, crescendo angina and acute coronary syndromes.

Recent epidemiological and observational studies have suggested a link between systemic inflammation and coronary artery disease. Infections by organisms such as *chlamydia pneumoniae* and *herpes simplex virus* type 1, appear to be associated with an increased risk of cardiovascular mortality [Roivanen *et al* 2000], and approximately 4% of bacteraemic patients will develop acute myocardial infarction within a month of infection [Valtonen *et al* 1993]. Increased cardiovascular mortality is also seen following respiratory tract infections [Meier *et al* 1998], severe illnesses requiring intensive care [Quartin *et al* 1997] and surgery [Mamode *et al* 1995]. Markers of systemic inflammation, such as C-reactive protein (CRP), serum amyloid A, interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), are elevated in patients with cardiovascular disease and are associated with an adverse prognosis and recurrent coronary events [Liuzzo *et al* 1994; Ridker *et al* 2000a; Biasucci *et al* 1996, 1999]. Moreover, in previously healthy individuals, elevated plasma CRP and IL-6 concentrations predict the development of cardiovascular disease [Ridker *et al*

1997, 2000b, 2000c]. Indeed, reflecting its anti-inflammatory action, the preventative benefits of aspirin in reducing cardiovascular risk are proportional to the plasma CRP concentration [Ridker *et al* 1997]. These data collectively suggest two patterns of association: a link between chronic inflammation and the slow process of atherogenesis, and an association between acute systemic inflammation and a transiently increased risk of an acute cardiovascular event.

The vascular endothelium plays a vital role in the control of blood flow, haemostasis, fibrinolysis and inflammation [Vane *et al* 1990], and impaired endothelial function is implicated in the pathogenesis of coronary artery disease. Tissue plasminogen activator is a fibrinolytic factor released from the endothelium through the translocation of a dynamic intracellular storage pool and regulates the degradation of intravascular fibrin [van den Eijnden-Schrauwen *et al* 1995]. If endogenous fibrinolysis is to be effective, then the rapid mobilisation of t-PA from the endothelium is essential because thrombus dissolution is much more effective if t-PA is incorporated during, rather than after, thrombus formation [Fox *et al* 1984; Brommer 1984]. However, in the presence of pro-inflammatory states or an imbalance in endogenous fibrinolysis, such microthrombi may propagate, ultimately leading to arterial occlusion and tissue infarction [Rosenberg & Aird 1999].

We have previously described an *in vivo* model to assess the acute release of t-PA in man [Newby *et al* 1997a] and demonstrated an association between t-PA release and endothelial dysfunction [Newby *et al* 1998b, 1999]. Hingorani and colleagues have also recently shown that acute inflammation causes dysfunction of endothelium-

dependent vasodilatation in humans [Hingorani *et al* 2000]. However, there have been no studies to assess directly the acute local fibrinolytic capacity following acute inflammation. The aim of this study is therefore to test the hypothesis that the acute fibrinolytic capacity is altered by a mild systemic inflammatory response generated by typhoid vaccination.

3.3 Methods

3.3.1 Subjects

Eight healthy non-smoking men aged between 20 and 27 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, vascular or coronary artery disease and none of the subjects had undergone typhoid vaccination in the previous year.

3.3.2 Drugs

An inflammatory response was generated with typhoid vaccination using *Salmonella typhi* capsular polysaccharide vaccine 0.025 mg (Typhim Vi, Aventis Pasteur MSD, Berkshire, United Kingdom). Pharmaceutical-grade bradykinin and acetylcholine (both from Clinalfa, Läufelfingen, Switzerland) and sodium nitroprusside (David Bull Laboratories, Faulding, United Kingdom) were administered following dissolution in saline. All solutions were freshly prepared on the day of study.

3.3.3 Study Design

S. typhi vaccine or saline placebo were injected into the deltoid muscle of the dominant arm at 8.30 AM in a randomised, balanced block, double-blind crossover manner at least two weeks apart. Previous reports have indicated that vaccination-

induced endothelial dysfunction is transient and resolves within thirty-two hours [Hingorani *et al* 2000].

Six hours after vaccination, and after a four-hour fast, strain gauges and cuffs were applied. The brachial artery of the non-dominant arm was cannulated and venous cannulae were inserted into both arms. Forearm blood flow was measured every six to ten minutes. After thirty minutes equilibration with saline infusion, intra-arterial bradykinin was administered at 40, 200, 1000 pmol/min for ten minutes at each dose, acetylcholine at 5, 10, 20 μg/min and sodium nitroprusside at 2, 4, 8 μg/min for six minutes at each dose. The drugs were separated by twenty minutes of saline infusion and administered in a randomised order that was kept constant for each individual. Venous samples were taken at baseline and during infusion of each bradykinin dose but not during sodium nitroprusside or acetylcholine infusion since they do not affect plasma t-PA or PAI-1 concentrations in this forearm model [Newby *et al* 1997a; Brown *et al* 1999]. White cell count, haematocrit, IL-6 and CRP were determined six hours after vaccination and haematocrit was repeated at the end of the forearm study.

3.3.4 Data Analysis and Statistics

Estimated net release of t-PA antigen and activity was defined previously [Newby *et al* 1997a] as the product of the infused forearm plasma flow (based on the mean haematocrit, Hct, and the infused forearm blood flow, FBF) and the concentration difference between the infused ($[t\text{-}PA]_{inf}$) and noninfused ($[t\text{-}PA]_{Noninf}$) arms. The area under the curve (AUC) was calculated for estimated net t-PA antigen and

activity release during bradykinin infusion. Data were examined, where appropriate, by analysis of variance (ANOVA) with repeated measures and 2-tailed paired Student's *t*-test using Excel 97 (Microsoft). All results are expressed as mean±SEM. Statistical significance was taken at the 5% level.

3.4 Results

3.4.1 Inflammatory Response

All subjects remained well throughout the study and reported no localised discomfort or systemic side effects following vaccination. Compared to placebo injection, there was a marked elevation in white cell count (11.1±0.5 *vs* 7.9±0.8×10^9/L; P=0.004, t-test) and plasma IL-6 concentrations (6.9±1.4 *vs* 1.6±0.4 pg/mL; P=0.01, t-test) six hours after typhoid vaccination, although serum CRP concentrations (1.8±1.2 *vs* 1.0±0.6 μg/mL; P=NS, t-test) and temperature (P=NS, t-test; Table 3.1) were unchanged.

Table 3.1 Baseline characteristics

	Vaccination	**Placebo**	
Temperature	37.0±0.1	36.9±0.1	°C
Systolic Blood Pressure	131±4	129±5	mmHg
Diastolic Blood Pressure	62±3	66±3	mmHg
Heart Rate	57±1	57±2	beats/min
Haematocrit	0.41±0.01	0.41±0.01	
Infused Forearm Blood Flow	3.0±0.6	2.8±0.6	mL/100 mL tissue/ min

3.4.2 Assessment of Endothelium-dependent Vasomotion

There were no significant effects of vaccination on heart rate, blood pressure, or baseline forearm blood flow. There were no significant changes in heart rate, blood pressure and non-infused forearm blood flow during drug infusion on either study day. Forearm blood flow increased in a dose-dependent manner during bradykinin, acetylcholine, and sodium nitroprusside infusions ($P<0.001$, ANOVA), but there was no change in the blood flow response following vaccination (P=NS, ANOVA; Figure 3.1).

3.4.3 Assessment of Fibrinolytic Activity

Following vaccination, there were no changes in baseline plasma t-PA and PAI-1 antigen concentrations or plasma t-PA activity concentration. Compared with the non-infused arm, bradykinin caused dose-dependent increases in plasma t-PA antigen and activity ($P<0.001$ for both, ANOVA) concentrations in the infused arm that was significantly higher following vaccination ($P<0.03$, ANOVA; Table 3.2). *S. typhi* vaccination caused a significant augmentation in the net release of t-PA antigen and activity ($P=0.016$ and $P=0.006$ respectively, ANOVA), and a two-three fold increase in the AUC for net t-PA antigen (37±12 vs 13±9 ng/100 mL/min; $P=0.14$, *t*-test) and activity (70±12 vs 36±8 IU/100 mL/min; $P=0.037$, *t*-test) release compared to placebo (Figure 3.2). Plasma PAI-1 concentrations did not change during bradykinin infusion (Table 3.2).

Table 3.2 Plasma t-PA and PAI-1 antigen and t-PA activity concentrations

	Typhoid Vaccination				Placebo Injection			
Bradykinin infusion, pmol/min	**0**	**40**	**200**	**1000**	**0**	**40**	**200**	**1000**
t-PA antigen (ng/mL)								
Noninfused Arm	2.3±0.3	2.7±0.5	2.9±0.5	3.5±0.5	2.8±0.6	3.2±0.8	3.6±0.7	4.4±0.8
Infused Arm	3.0±0.5	3.2±0.5	3.9±0.5	6.7±0.7*	2.8±0.6	3.0±0.7	3.8±0.8	6.1±0.9*
Concentration differences between forearms	0.7±0.4	0.5±0.4	1.0±0.6	3.3±0.8§	0.0±0.2	-0.1±0.4	0.2±0.3	1.7±0.5§‡
t-PA activity (IU/mL)								
Noninfused Arm	1.3±0.2	1.4±0.2	1.7±0.2	2.6±0.4	1.1±0.2	1.2±0.2	1.5±0.2	2.5±0.5
Infused Arm	1.1±0.2	1.6±0.3	3.3±0.6	9.9±1.2*	1.0±0.2	1.3±0.2	2.5±0.5	6.5±1.3*†
Concentration differences between forearms	-0.2±0.1	0.2±0.1	1.6±0.5	7.4±1.0*	-0.1±0.1	0.1±0.1	0.9±0.5	3.9±1.0*‡
PAI antigen (ng/mL)								
Noninfused Arm	17±4			16±2	15±2			16±3
Infused Arm	18±5			14±2	16±3			15±2
Concentration differences between forearms	1.3±2.4			-1.2±1.3	0.4±0.8			-0.5±1.7

One-way ANOVA: *$P<0.001$, §$P<0.01$ (dose response)
Two-way ANOVA: †$P=0.026$, ‡$P=0.01$ (vaccination *vs* placebo); $P<0.001$ for all (dose response)

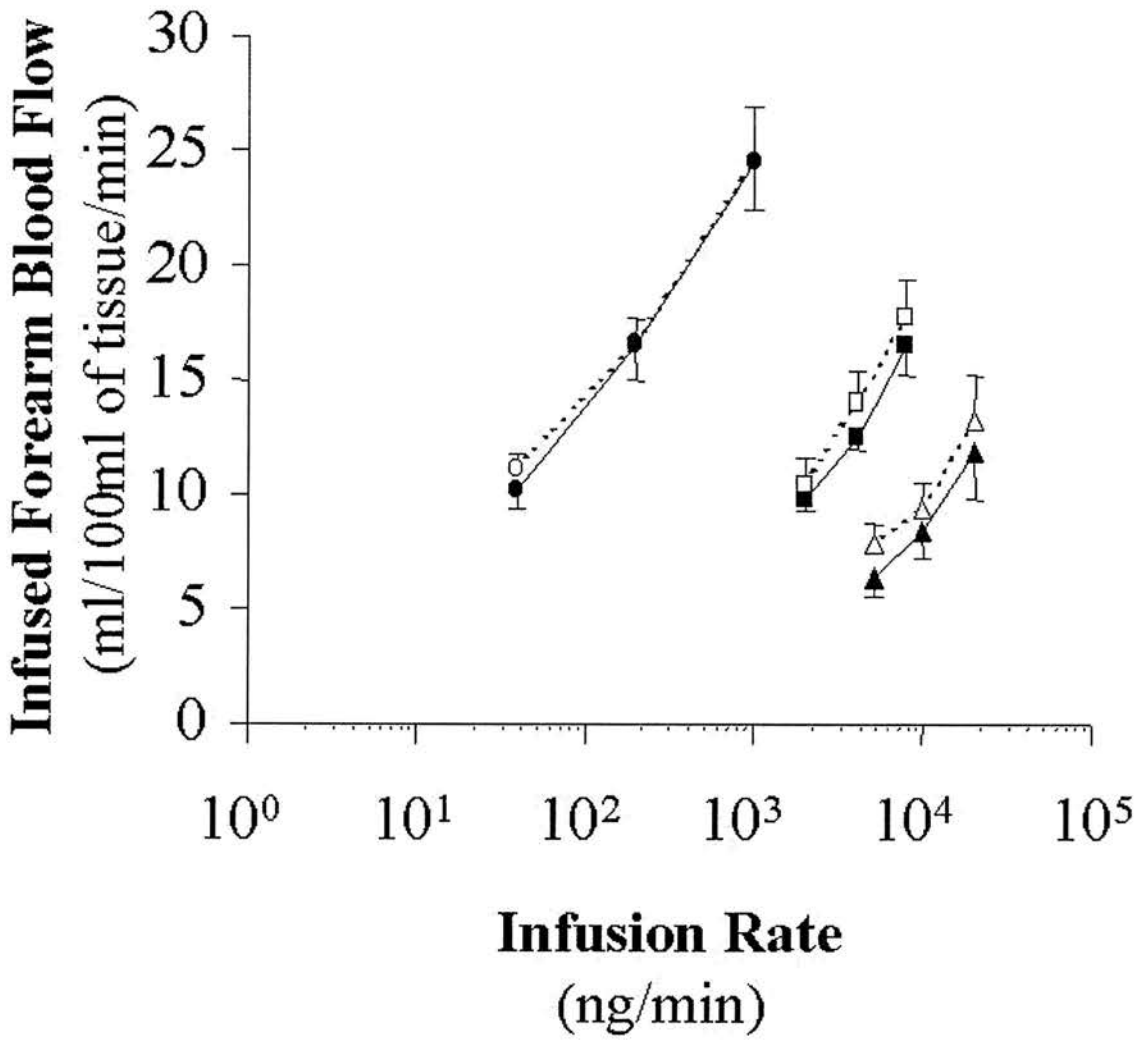

Figure 3.1
Infused forearm blood flow during bradykinin (circles), acetylcholine (triangles) and sodium nitroprusside (squares) infusions in subjects who were administered typhoid vaccination (closed symbols) and saline placebo (open symbols).
ANOVA $P<0.001$ for all (dose response)

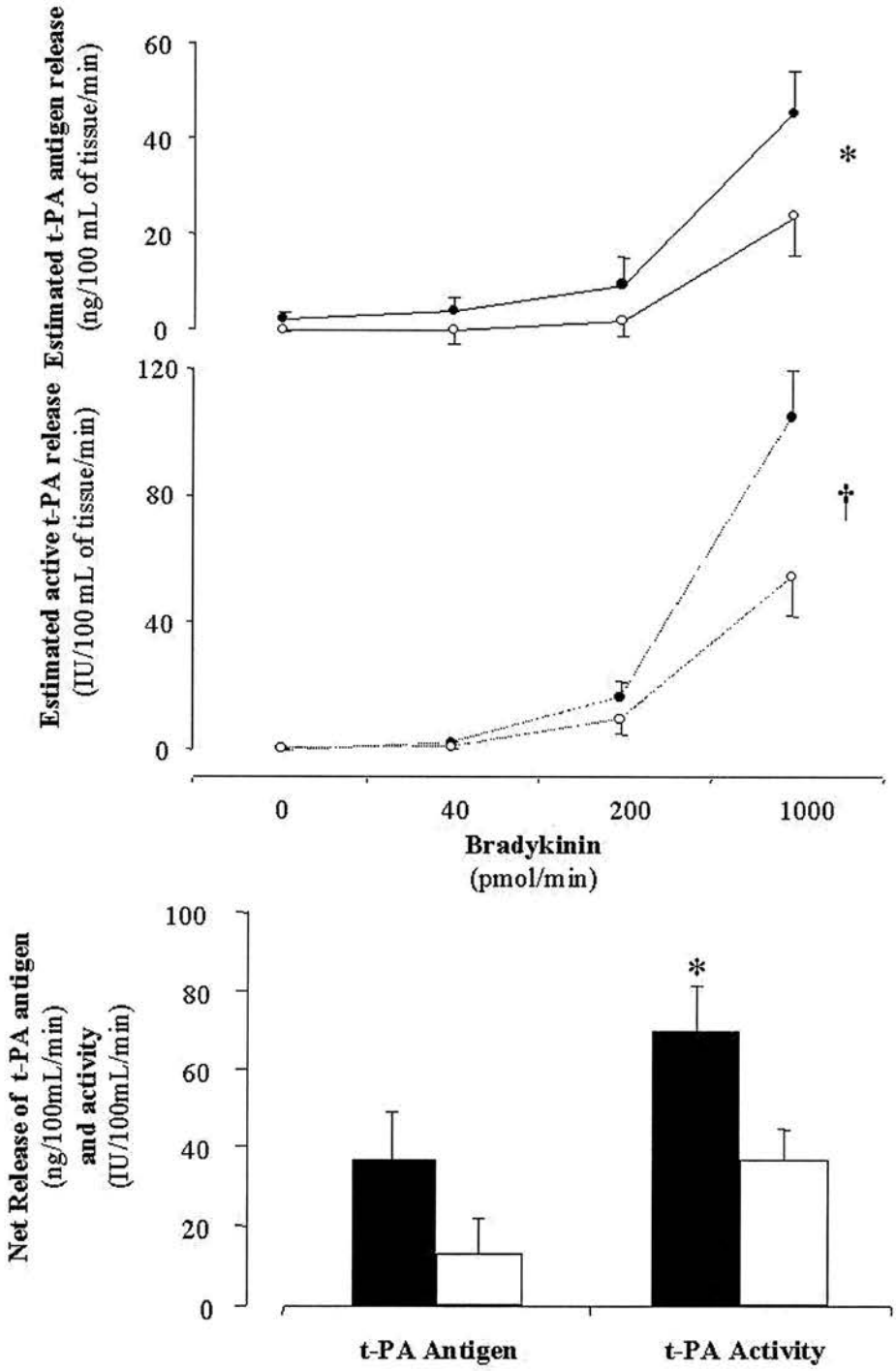

Figure 3.2
Estimated net release of t-PA antigen (solid line) and activity (dashed line) in subjects who were administered typhoid vaccination (closed circles and bars) and saline placebo (open circles and bars). Lower panel represents area under the curve for the response.

ANOVA *P<0.05; †P=0.006 (vaccination vs placebo)

t-test ‡P<0.05 (vaccination vs placebo)

3.5 Discussion

We have demonstrated that an acute mild systemic inflammatory stimulus causes potentiation of bradykinin-induced t-PA release. We conclude that acute systemic inflammation enhances local endothelial t-PA release in man. This may reflect an adaptive mechanism of the vascular endothelium to augment its fibrinolytic response under circumstances of acute inflammation.

3.5.1 Endothelium-dependent Vasomotion

We assessed basal and stimulated forearm blood flow following intra-arterial infusions of the endothelium-dependent vasodilators, bradykinin and acetylcholine, and the endothelium-independent vasodilator sodium nitroprusside, six to eight hours after vaccination. Bradykinin and acetylcholine have both been widely used to investigate the function of vascular endothelium. Impaired arterial vasodilatory response to endothelium-dependent agonists has been shown in patients with hypertension [Panza *et al* 1990], diabetes mellitus [Calver *et al* 1992] and hypercholesterolemia [Chowienczyk *et al* 1992]. Hingorani and colleagues [Hingorani *et al* 2000] have previously reported that *S. typhi* vaccination generated an inflammatory response that was associated with a temporary suppression of endothelium-dependent vasodilatation in the forearm circulation of six healthy volunteers. Although we applied a similar protocol to their study and included overlapping doses of both bradykinin and acetylcholine, we did not replicate their findings of impaired forearm endothelium-dependent vasodilatation following vaccination at 6-8 hours (Figure 3.1). This discrepancy may be partly explained by

the variability in vasodilatory response with acetylcholine [Chowienczyk *et al* 1994] and the higher maximal vasodilator dose used in our studies, although we have used a larger sample size and employed a double-blind randomised placebo-controlled crossover trial.

3.5.2 Endogenous Fibrinolysis

Tissue plasminogen activator, the key enzyme in the initiation of fibrinolysis, is synthesised in endothelial cells and stored in small, dense vesicles. It is secreted both basally and in response to thrombin and several vasoactive agents through a calcium-dependent and G protein-coupled pathway [Emeis *et al* 1997]. The regulated endogenous release of t-PA plays a major role in the defense against intravascular thrombosis, especially in the coronary circulation [Rosenberg & Aird 1999]. Bradykinin is a vasoactive peptide and potent stimulant for the acute release of t-PA from the vasculature [Brown *et al* 1997, 1999; Labinjoh *et al* 2000, 2001] and is produced locally through activation of the kallikrein-kinin system on the surface of endothelial cells [Rojkjaer & Schmaier 1999]. In the present study, bradykinin-induced t-PA antigen and activity release was augmented two-three fold following typhoid vaccination in the absence of systemic haemodynamic effects (Figure 3.2).

The underlying mechanisms for our findings are unknown. Inflammation is recognised to induce a protective response towards tissue injury, and it functions as part of normal host surveillance mechanisms. Various compounds associated with the inflammatory response, including histamine, thrombin and endotoxin, have been shown to increase cellular t-PA transcription and expression [Dichek & Quertermous

1989; Levin & Santell 1988]. However, we did not observe an increase in the basal plasma concentrations of either t-PA or PAI-1. The inflammatory stimulus, therefore, appears to augment specifically the storage or acute release of t-PA rather than a generalised upregulation of protein synthesis and basal secretion. This may be mediated by pro-inflammatory cytokines, such as IL-6, that modulate cellular activation leading to alterations in endothelial function. In particular, molecular and pharmacological evidence supports the role of bradykinin B2 receptors in the acute phase of inflammation, and upregulation of B2 receptors may account for the potentiation of bradykinin-induced t-PA release.

3.5.3 Clinical Implications

The augmentation of acute t-PA release following typhoid vaccination suggests that mild acute inflammation may induce anti-thrombotic properties in the forearm circulation. This may represent an adaptive response to inhibit intravascular thrombus deposition under circumstances of acute vascular inflammation. This observation is consistent with the increase in endogenous fibrinolysis in systemic inflammation induced by experimental endotoxaemia in healthy subjects [van der Poll *et al* 1997]. However, in susceptible individuals, such as those with ischaemic heart disease and chronic inflammation, this adaptive and protective acute response may fail or become depleted, leading to thrombus propagation and vessel occlusion. The fibrinolytic response to acute systemic inflammation in patients with ischaemic heart disease and the influence of anti-inflammatory therapies, such as aspirin, now needs to be established. Indeed, recent evidence has suggested that aspirin pre-

treatment is able to reverse inflammation-induced endothelial dysfunction [Kharbanda *et al* 2002].

Epidemiological studies have demonstrated an association between the risk of future cardiovascular events and both plasma inflammatory markers [Ridker *et al* 1997, 2000b, 2000c] and fibrinolytic factors [Hamsten *et al* 1985]. The current observations are, therefore, consistent with the suggestion that elevated plasma t-PA concentrations may provide a marker of vascular inflammation. Irrespective of whether these common associations are partly or wholly explained by inflammation-induced t-PA release, understanding the regulation of both acute and chronic t-PA release will have important clinical implications and may help to develop more effective strategies in the management of atherosclerotic disease.

3.5.4 Study Limitations

There are some limitations to our study. We administered the typhoid vaccination in the deltoid muscle of the control dominant arm rather than the gluteus muscle. However, as blood flow was assessed in the forearm and intra-arterial vasodilators were administered in the contralateral non-dominant arm, it would be highly unlikely that the site of vaccination would have affected the response in the infused forearm. It would also have been preferable to assess the vascular responses immediately before and 6-8 hours after vaccination on the same day. However, this would require repeated arterial cannulations within the same day and we have previously demonstrated that endothelium-dependent vasodilatation and t-PA release is reproducible when performed at least one week apart [Newby *et al* 1997b, 2002].

The study subjects are healthy and young, and we acknowledge that the response in older subjects may be quite different. Finally, we studied peripheral vascular function and thus these findings may not be directly applicable to other vascular beds. However, endothelial dysfunction is often a generalised process, and we have previously shown [Newby *et al* 1999, 2001] consistent endogenous fibrinolytic responses between the forearm and coronary circulation.

3.5.5 Conclusion

In conclusion, we have demonstrated that mild inflammation generated by typhoid vaccination resulted in a significant potentiation of bradykinin-induced t-PA release from the vascular endothelium. Additional work is now required to determine the underlying mechanism and to assess the effects of acute and chronic inflammation on endogenous fibrinolysis in health and disease.

Chapter 4

The Direct Effects of Inflammatory Cytokines and Endotoxin on Local Endothelial Vasomotor and Fibrinolytic Function in Man

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Intra-arterial Tumor Necrosis Factor-alpha Impairs Endothelium-Dependent Vasodilatation and Stimulates Local Tissue Plasminogen Activator Release In Humans.

Arterioscler Thromb Vasc Biol. 2003;**23**:695-701.

4.1 Summary

Systemic and vascular inflammation contribute to the pathogenesis of cardiovascular disease, potentially through the actions of pro-inflammatory cytokines. The aims of this study were to assess the direct effects of local intra-arterial tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and endotoxin on blood flow and endogenous tissue-type plasminogen activator (t-PA) release *in vivo* in man. In a double-blind randomised placebo-controlled trial, blood flow, and plasma cytokine and fibrinolytic factor concentrations were measured in both forearms of healthy male volunteers using venous occlusion plethysmography and blood sampling. Ten subjects received intra-brachial infusions of TNF-α (80 or 240 ng/min), IL-6 (30 ng/min), endotoxin (100 pg/min) or saline placebo. Eight further subjects received intra-brachial bradykinin, acetylcholine and sodium nitroprusside, following pre-treatment with TNF-α (80 ng/min). TNF-α, but not IL-6, endotoxin or placebo, caused a gradual and sustained ~20-fold increase in plasma t-PA concentrations ($P<0.001$) that was associated with elevated plasma IL-6 concentrations ($P<0.05$) but without an effect on blood flow or plasminogen activator inhibitor type 1 antigen. Compared to placebo, TNF-α pre-treatment impaired bradykinin- and acetylcholine-induced vasodilatation ($P<0.03$), and resulted in a doubling of bradykinin-induced t-PA release ($P<0.05$). We conclude that intra-arterial TNF-α causes an acute local vascular inflammation that is associated with impaired endothelium-dependent vasomotion as well as sustained and substantial increase in endothelial t-PA release. TNF-α has potentially both adverse vasomotor and beneficial profibrinolytic effects on endothelial function.

4.2 Introduction

There is emerging evidence that systemic inflammation plays a major role in the pathogenesis of cardiovascular disease. The pro-inflammatory cytokines, TNF-α and IL-6, in particular, have been implicated in the initiation and maintenance of systemic and vascular inflammation associated with atherosclerosis and coronary artery disease. Indeed, plasma concentrations of these cytokines are elevated in patients with ischaemic heart disease [Biasucci *et al* 1999; Ridker *et al* 2000a], and have been shown to predict the future risk of myocardial infarction in apparently healthy individuals [Ridker *et al* 2000b].

The vascular endothelium plays a vital role in the control of blood flow, haemostasis, fibrinolysis and inflammation, and changes in endothelial function may underlie the association between inflammation and the risk of cardiovascular disease. Tissue-type plasminogen activator is a fibrinolytic factor released from the endothelium and lyses intravascular fibrin [van den Eijnden-Schrauwen *et al* 1995]. If endogenous fibrinolysis is to be effective, then the rapid mobilisation of t-PA from the endothelium is essential because thrombus dissolution is much more effective if t-PA is incorporated during, rather than after, thrombus formation [Fox *et al* 1984]. However, in the presence of pro-inflammatory states or an imbalance in endogenous fibrinolysis, intravascular thrombus may propagate, ultimately leading to arterial occlusion and tissue infarction [Rosenberg & Aird 1999].

TNF-α and endotoxin have been reported to induce local vascular inflammation and impair endothelium-dependent vasodilatation in the venous circulation of man [Bhagat & Vallance 1997]. Although mild systemic inflammation has also been shown to alter endothelial function [Hingorani *et al* 2000], the underlying mechanisms for these observations have not been elucidated and the direct *in vivo* effects of cytokines and inflammatory stimuli on local arterial endothelial vasomotor and fibrinolytic function are unknown.

The aims of this study were to investigate the acute effects of local intra-arterial inflammatory cytokines (IL-6, TNF-α) and bacterial endotoxin (lipopolysaccharide, LPS) exposure on vasomotor function and endothelial t-PA release *in vivo* in man.

4.3 Methods

4.3.1 Subjects

Eighteen healthy non-smoking men aged between 21 and 25 years participated in the study which was undertaken with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki and the written informed consent of each subject. None of the subjects had infective illnesses or received medication in the week before study.

4.3.2 Cytokines and Drugs

TNF-α (Knoll Pharmaceuticals, Germany) [Janik *et al* 1999], IL-6 (Novartis Pharma AG, Basel, Switzerland) [Tsigos *et al* 1997], LPS (lot G-1, USPCI, Rockville, Maryland, USA) [Pernerstorfer *et al* 1999], bradykinin (Clinalfa, Läufelfingen, Switzerland), acetylcholine (Cibavision Ophthalmics, Southampton, Untied Kingdom) and sodium nitroprusside (David Bull Laboratories, Faulding, United Kingdom) were administered following dissolution in 0.9% saline. TNF-α, IL-6 and LPS were prepared as stock solutions and stored at -80 °C in aliquots. All other drugs were freshly prepared on the study day.

4.3.3 Study Design

Subjects rested recumbent, strain gauges and cuffs were applied, and venous cannulae were inserted into both antecubital fossae. The brachial artery of the non-dominant arm was cannulated under local anaesthesia. Venous sampling was performed on both arms for cytokine, coagulation and fibrinolytic factor assays

4.3.3.1 Protocol 1: *Cytokine and Endotoxin Administration*

In a randomised double-blind study, ten subjects attended on three occasions at least one week apart and saline was infused for 30 min to allow time for equilibration. Subjects were then randomised to receive intra-arterial infusions of low dose TNF-α (80 ng/min; n=6), high dose TNF-α (240 ng/min; n=6), IL-6 (30 ng/min; n=6), LPS (100 pg/min; n=6) or saline placebo (1 mL/min; n=6) over 60 min. This was followed by a further 60 min saline washout infusion. Venous samples were obtained at baseline, 10, 20, 40 and 60 min during drug infusion, and 30, 60 and 180 min after cessation of drug infusion (Figure 4.1). Cytokine and LPS doses were chosen to achieve local concentrations comparable to healthy volunteer studies [Bhagat & Vallance 1997; Pernerstorfer *et al* 1999; van der Poll *et al* 1991], and those seen in cardiovascular diseases [Biasucci *et al* 1999; McMurray *et al* 1991].

4.3.3.2 Protocol 2: *Effect of TNF-α on Endothelial Function*

Eight subjects attended on two occasions at least one week apart in a randomised, double-blind placebo-controlled crossover trial. They received an intra-arterial infusion of either TNF-α (80 ng/min) or saline placebo (1 mL/min) over 60 min. After a further 60 min saline infusion, intra-arterial bradykinin was administered at 100, 300, 1000 pmol/min for 10 min at each dose, acetylcholine at 5, 10, 20 μg/min and sodium nitroprusside at 2, 4, 8 μg/min for 5 min at each dose (Figure 4.2). Infusions of the vasoactive drugs were separated by 15 min saline washout infusions. Venous samples were obtained at baseline, after 60 min of TNF-α/placebo infusion, before and during each dose of bradykinin and 15 min after cessation of bradykinin infusion. Venous samples were not obtained during acetylcholine or sodium

nitroprusside infusion since they do not affect plasma t-PA or plasminogen activator inhibitor type 1 (PAI-1) concentrations in this forearm model [Newby *et al* 1997a; Brown *et al* 1999; Jern *et al* 1994].

4.3.4 Data Analysis and Statistics

Estimated net t-PA antigen and activity release has been defined previously in Chapter 2.5.2. Because basal t-PA concentrations were altered by pre-treatment with TNF-α, t-PA antigen and activity release during bradykinin infusion was calculated by subtracting the mean t-PA release before, and 15 min after cessation of bradykinin infusion. Data were examined, where appropriate, by analysis of variance (ANOVA) with repeated measures and two-tailed paired Student's *t*-test. All results are expressed as mean±SEM. Statistical significance was assigned at the 5% level.

Drugs: TNF-α (80 ng/min)
TNF-α (240 ng/min)
IL-6 (30 ng/min)
Lipopolysaccharide (100 pg/min)
Saline Placebo (1 mL/min)

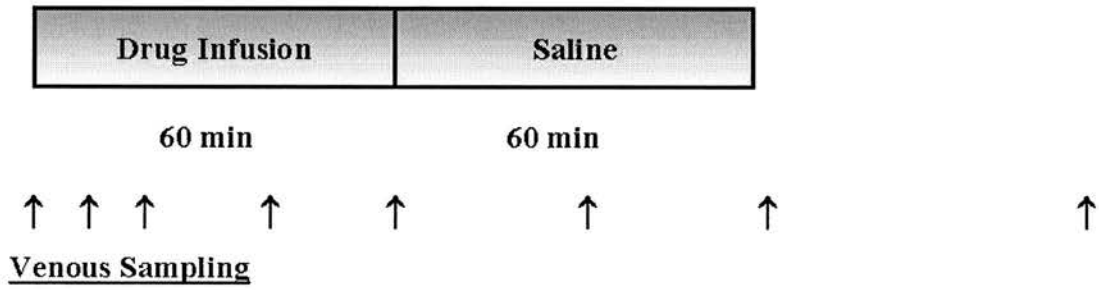


Figure 4.1
Study design of Protocol 1: Cytokine and Endotoxin Administration.

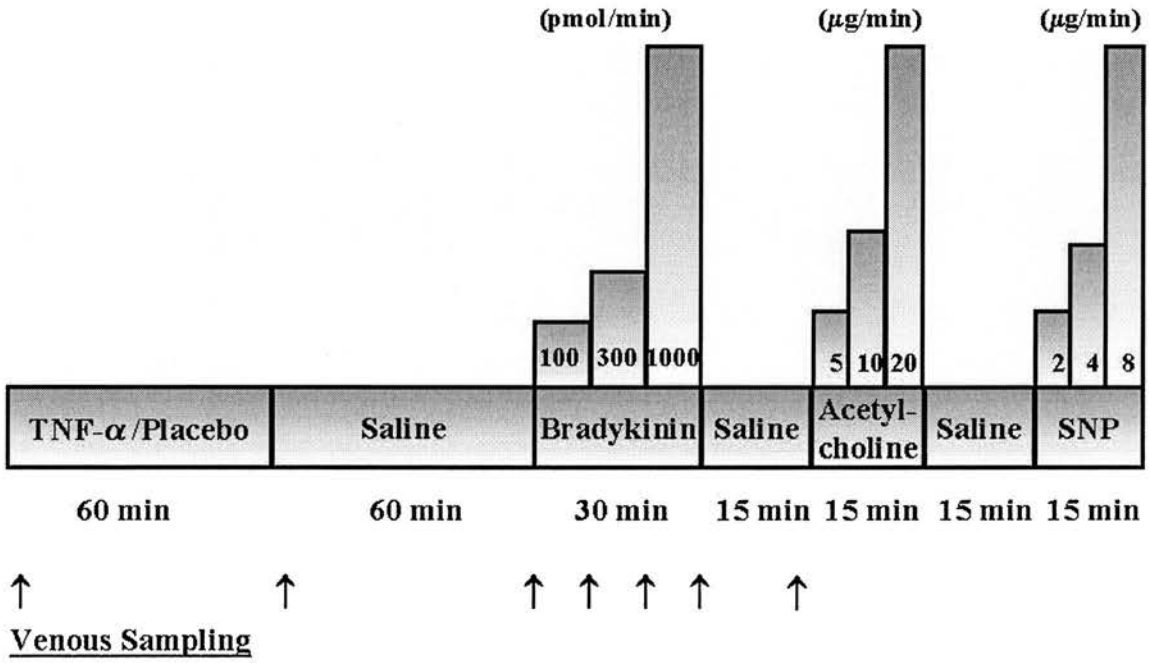


Figure 4.2
Study design of Protocol 2: Effect of Tumour Necrosis Factor-α on Endothelial Function.
SNP: Sodium Nitroprusside.

4.4 Results

All subjects remained well throughout the study and reported no adverse effects. There were no effects on haematocrit, body temperature or white cell count throughout all studies (Tables 4.1 and 4.2).

4.4.1 Protocol 1: Cytokine and Endotoxin Administration

4.4.1.1 Cytokine Assays

Plasma TNF-α concentrations increased from 1±0 and 2±1 pg/mL to 539±71 and 1164±41 pg/mL in the infused arm ($P<0.001$ for both), and to 20±2 and 62±8 pg/mL in the non-infused arm ($P<0.001$ for both) during 80 and 240 ng/min of TNF-α respectively (Figure 4.3). This was accompanied by a gradual increase in plasma IL-6 concentrations (Figure 4.3).

In the infused arm, IL-6 infusion increased plasma IL-6 concentrations from 2±1 to 14±3 pg/ml ($P=0.01$), and LPS infusion increased plasma TNF-α and IL-6 concentrations from 1±3 to 7±1 pg/mL ($P=0.01$) and from 1±0 to 6±2 pg/mL ($P=0.02$) respectively.

4.4.1.2 Haemodynamic Effects

Intra-arterial saline placebo, TNF-α, IL-6 and LPS infusions had no effect on heart rate, blood pressure or forearm blood flow throughout all studies (Tables 4.1 and 4.3).

4.4.1.3 Fibrinolytic and Haemostatic Assays

There were no changes in plasma t-PA antigen concentrations during IL-6, LPS or saline placebo infusions (Table 4.4).

Plasma t-PA antigen and activity concentrations increased in the infused arm by up to 20-fold following both low and high dose TNF-α infusions ($P<0.001$; Figure 4.4). Plasma t-PA concentrations increased slowly, being detectable at 20 min and peaking at 60 min of infusion. Thereafter, plasma t-PA concentrations fell but remained elevated 4 hours after initiation of the infusion with an apparently stable elevation between 2 and 4 hours.

Plasma PAI-1 and von Willebrand factor antigen concentrations were unchanged throughout all studies although plasma PAI-1 activity was reduced in the infused arm only following high dose TNF-α infusion ($P=0.003$; Table 4.5).

Table 4.1
Protocol 1: Temperature, White cell count, Haematocrit, Heart rate and Blood pressure During Cytokine and Endotoxin Infusion

	TNF-α 80 ng/min	TNF-α 240 ng/min	IL-6 30 ng/min	LPS 100 pg/min	Saline 1 ml/min
Temperature, °C					
Base	36.5±0.1	36.5±0.0	36.5±0.1	36.5±0.1	36.5±0.1
2 hours	36.5±0.1	36.7±0.1	36.4±0.1	36.6±0.1	36.4±0.1
4 hours	36.3±0.1	36.4±0.1	36.4±0.1	36.3±0.1	36.3±0.2
White Cell Count, $x10^9/L$					
Baseline	5.2±0.6	4.9±0.5	4.9±0.4	4.9±0.4	5.7±0.4
2 hours	4.9±0.5	5.6±0.4	5.6±0.7	5.1±0.5	6.0±0.5
4 hours	6.0±0.5	7.1±0.9	5.9±0.4	6.8±0.9	6.6±0.3
Haematocrit					
Base	0.39±0.01	0.38±0.01	0.39±0.01	0.38±0.01	0.39±0.01
2 hours	0.39±0.00	0.37±0.01	0.39±0.01	0.38±0.01	0.38±0.01
Heart Rate, bpm					
Baseline	51±4	55±4	54±6	59±6	56±2
1 hour	54±4	60±4	52±5	57±6	61±3
2 hours	55±4	59±4	55±5	58±5	61±4
Arterial Blood Pressure, mmHg					
Baseline	84±3	89±3	86±2	87±3	86±3
1 hour	87±2	93±2	84±2	89±3	90±3
2 hours	93±1	88±4	89±3	89±5	87±3

Table 4.2
Protocol 2: White Cell Count and Haematocrit During TNF-α Infusion

	TNF-α 80 ng/min	Saline 1 ml/min
White Cell Count, x10^9/L		
Baseline	5.6±0.8	5.0±0.2
2 hours	4.7±0.5	5.5±0.3
Haematocrit		
Pre-bradykinin infusion	0.40±0.01	0.41±0.01
Post-bradykinin infusion	0.41±0.00	0.41±0.01

Table 4.3
Protocol 1: Forearm blood flow (FBF) during TNF-α, IL-6, LPS and Saline infusions

	TNF-α 80 ng/min	TNF-α 240 ng/min	IL-6 30 ng/min	LPS 100 pg/min	Saline 1 mL/min
FBF (mL/100 mL/min)					
Infused Arm					
Baseline	2.2±0.2	2.6±0.6	2.4±0.1	2.5±0.3	2.2±0.3
1 hour	1.9±0.3	2.4±0.9	1.9±0.2	2.3±0.3	2.0±0.3
2 hours	1.8±0.3	2.1±0.7	1.8±0.2	2.0±0.3	1.7±0.3
Non-infused Arm					
Baseline	2.2±0.4	2.6±0.5	2.0±0.2	2.3±0.4	1.8±0.2
1 hour	2.6±0.5	2.6±0.8	2.0±0.3	2.0±0.3	1.7±0.2
2 hours	2.9±0.7	2.4±0.5	1.8±0.2	1.8±0.2	1.9±0.3

Table 4.4
Protocol 1: Plasma t-PA Antigen Concentrations During IL-6, LPS and Saline Infusion

	IL-6 (30 ng/min)		LPS (100 pg/min)		Saline (1 ml/min)	
	Infused Arm	**Non-infused**	**Infused Arm**	**Non-infused**	**Infused Arm**	**Non-infused**
T-PA antigen (ng/min)						
0 min	3.8±0.5	4.7±1.1	4.1±1.0	3.9±0.9	4.8±0.7	4.7±0.8
60 min	3.8±0.7	4.2±0.7	3.6±0.8	3.1±0.8	4.5±0.6	4.7±0.7
120 min	3.8±0.6	3.2±0.6	3.2±0.6	3.3±0.6	4.1±0.6	4.4±0.8
240 min	3.8±0.7	3.1±0.5	3.9±0.7	3.5±0.7	4.1±0.6	4.0±0.7

Table 4.5
Protocol 1: Plasma plasminogen activator inhibitor type 1 (PAI-1) antigen and activity concentrations during TNF-α infusion

	TNF-α (80 ng/min)		TNF-α (240 ng/min)	
	Infused	**Non-infused**	**Infused**	**Non-infused**
PAI-1 antigen (ng/mL)				
0 min	17±4	19±5	23±5	24±5
60 min	18±4	20±4	25±6	26±8
120 min	14±3	16±3	18±5	24±5
240 min	15±4	14±3	21±5	36±17
PAI-1 activity (IU/mL)				
0 min	12±2	11±1	10±2	9±2
60 min	9±2	10±2	5±2*	12±3
120 min	11±1	14±3	6±1*	10±2
240 min	8±1	11±2	4±1*	12±1

*P=0.003, ANOVA, infused *vs* non-infused arm

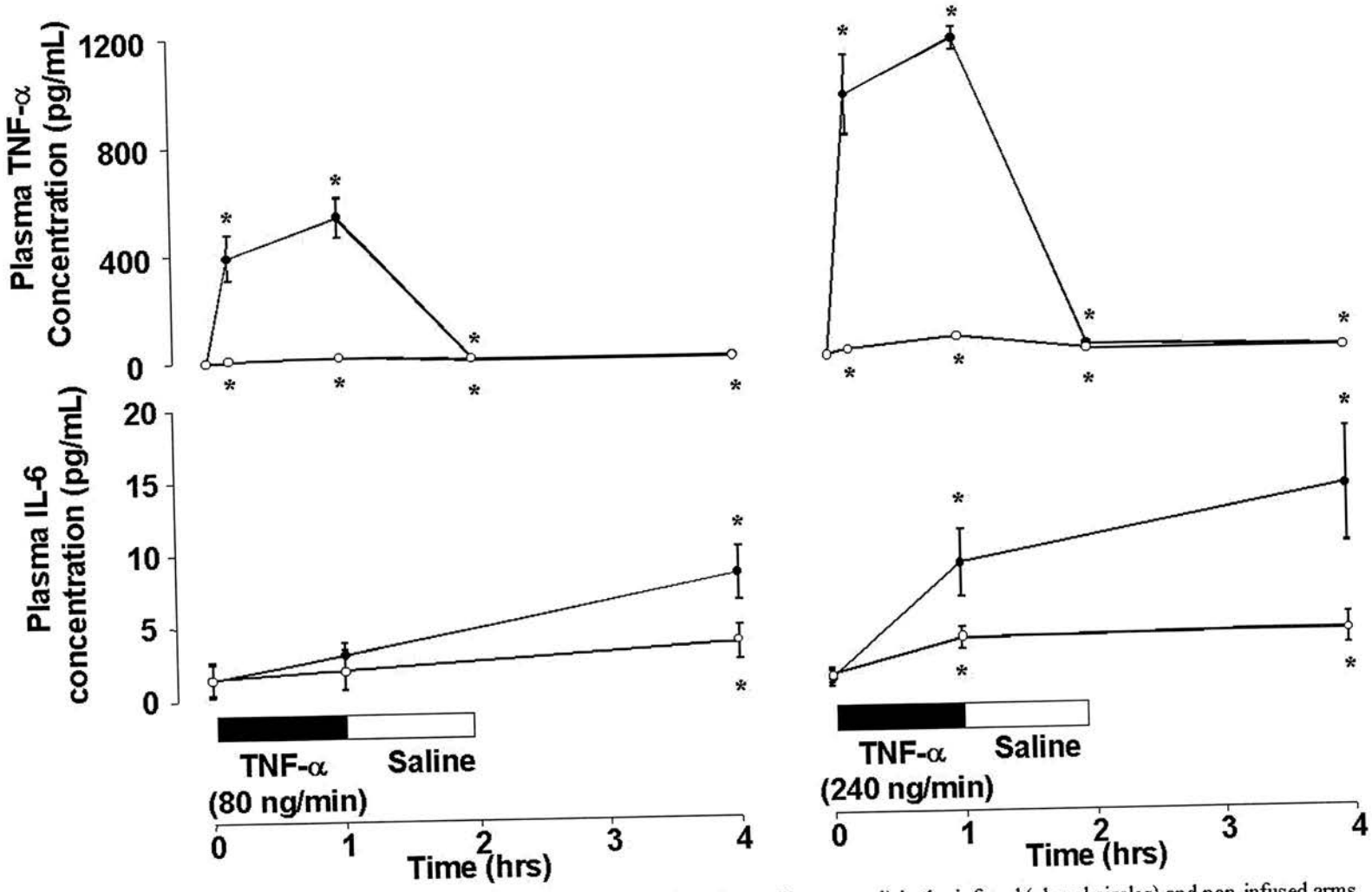


Figure 4.3 Protocol 1: Plasma concentrations of TNF-α (upper panel) and IL-6 (lower panel) in the infused (closed circles) and non-infused arms (open circles), following 1 hour intra-arterial infusion of TNF-α at 80 ng/min (left panel), and 240 ng/min (right panel). $P<0.01$ (ANOVA) for all responses except IL-6 concentrations in non-infused arm. $P<0.05$ (ANOVA, infused *vs* non-infused arm) for all responses. *$P<0.05$ (paired *t*-test; *vs* baseline)

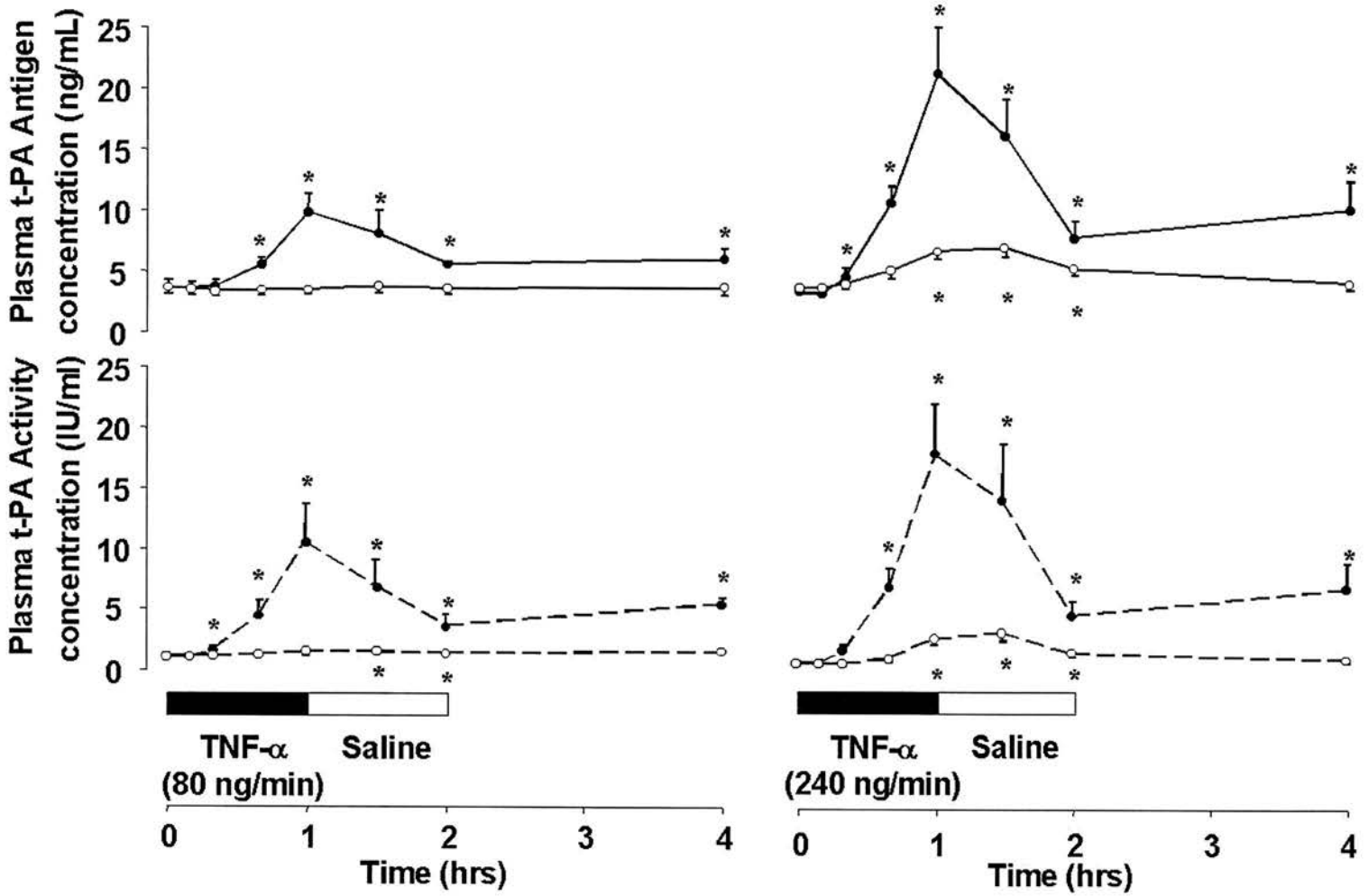


Figure 4.4 Protocol 1: Plasma concentrations of t-PA antigen (solid lines) and activity (dashed lines) in the infused (closed circles) and non-infused arms (open circles), following 1 hour intra-arterial infusion of TNF-α at 80 ng/min (left panel), and 240 ng/min (right panel). $P<0.001$ (ANOVA) for all responses except non-infused arm following TNF-α 80 ng/min infusion. $P<0.001$ (ANOVA, infused *vs* non-infused arm) for all responses. * $P<0.05$ (paired *t*-test; *vs* baseline)

4.4.2 Protocol 2: Effects of TNF-α on Endothelial Function

4.4.2.1 Cytokine Assays

Intra-arterial TNF-α infusion (80 ng/min) increased plasma TNF-α concentrations in the infused and non-infused arm from 2±1 and 2±1 pg/mL to 561±108 and 15±1 pg/mL respectively ($P<0.001$ for both).

4.4.2.2 Haemodynamic Effects

Intra-arterial TNF-α or placebo infusion had no significant effects on heart rate, blood pressure or basal forearm blood flow up to 2 hours after commencement of the infusion (P=NS for both). Forearm blood flow increased in a dose-dependent manner during bradykinin, acetylcholine and sodium nitroprusside infusions ($P\leq0.02$; Figure 4.5). Compared to placebo, TNF-α infusion caused impaired endothelium-dependent vasodilatation to bradykinin ($P=0.029$) and acetylcholine ($P=0.01$) but had no effect on endothelium-independent vasodilatation to sodium nitroprusside ($P=0.43$; Figure 4.5).

4.4.2.3 Fibrinolytic Assays

Plasma t-PA antigen and activity concentrations increased from 3.5±0.6 to 8.5±1.6 ng/mL ($P=0.004$), and from 1.1±0.3 to 9.2±1.9 IU/mL ($P=0.003$) respectively after 60 min of TNF-α infusion. Again, this rise was sustained with an apparently stable elevation between 2 and 3 hours i.e. 1 to 2 hours after cessation of TNF infusion (Figure 4.6). Plasma t-PA antigen and activity concentrations were unchanged in the non-infused arm and during placebo infusion.

Plasma t-PA antigen and activity concentrations increased in a dose-dependent manner during bradykinin infusion ($P \leq 0.01$). Compared to placebo, pre-treatment with TNF-α resulted in higher plasma t-PA antigen and activity concentrations during bradykinin infusion (11.3±0.8 *vs* 6.8±0.5 ng/mL and 16.5±3.9 *vs* 6.6±2.0 IU/mL at peak bradykinin dose; $P<0.002$) (Figure 4.6) and a marked augmentation in the estimated net release of t-PA antigen and activity ($P=0.018$ and $P=0.037$ respectively; Figure 4.7). For the 30 min bradykinin dose-response infusion, pre-treatment with TNF-α resulted in 83% and 132% increases in area under the curve for t-PA antigen and activity release ($P \leq 0.05$).

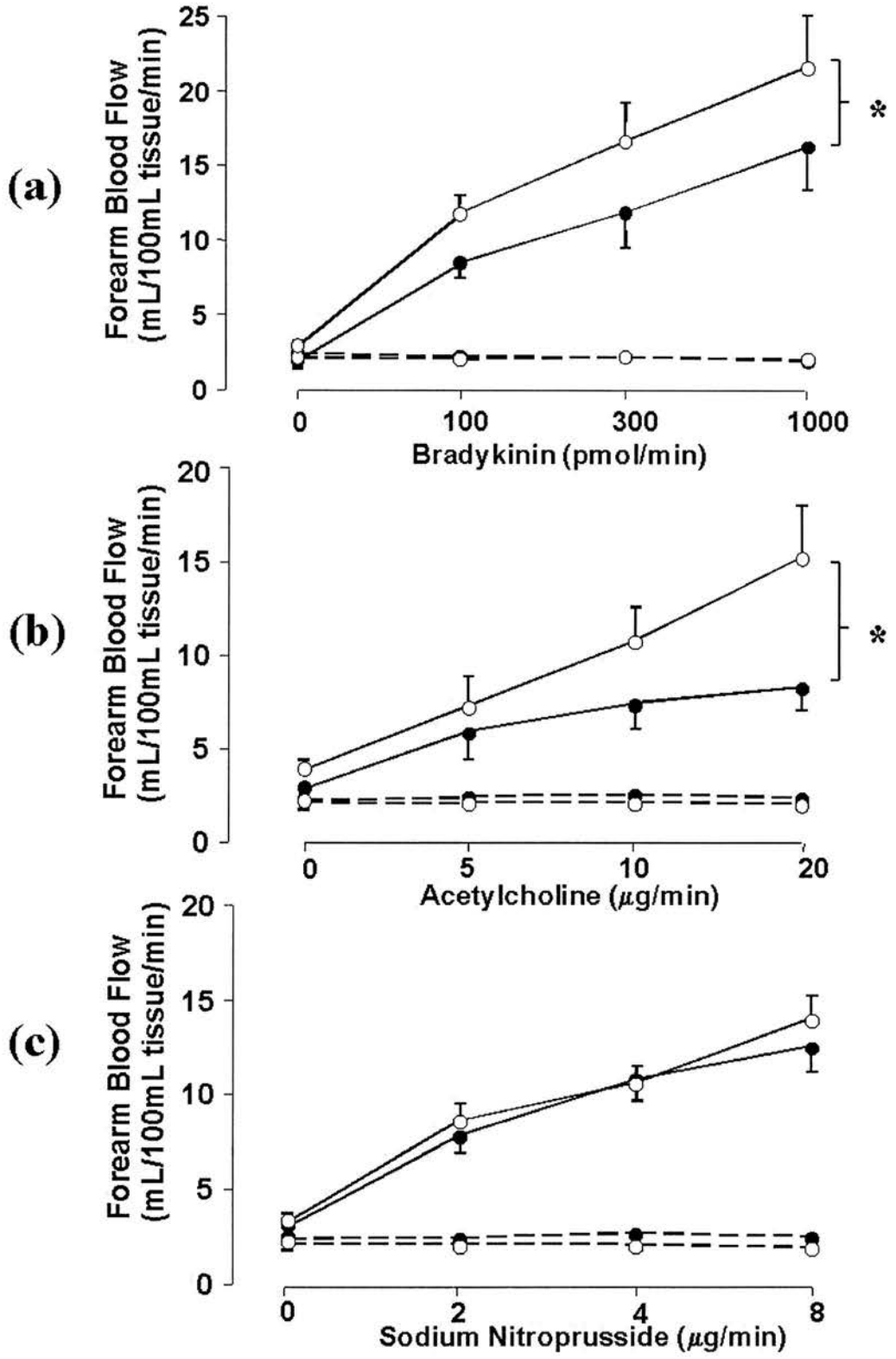


Figure 4.5 Protocol 2: Infused (solid line) and non-infused (dashed line) forearm blood flow responses to incremental doses of (a) bradykinin, (b) acetylcholine and (c) sodium nitroprusside in subjects who were pre-treated with TNF-α (closed circles) or saline placebo (open circles). $P \leq 0.02$ (ANOVA) for all infused arm responses. *$P < 0.03$ (ANOVA, TNF-α *vs* saline placebo)

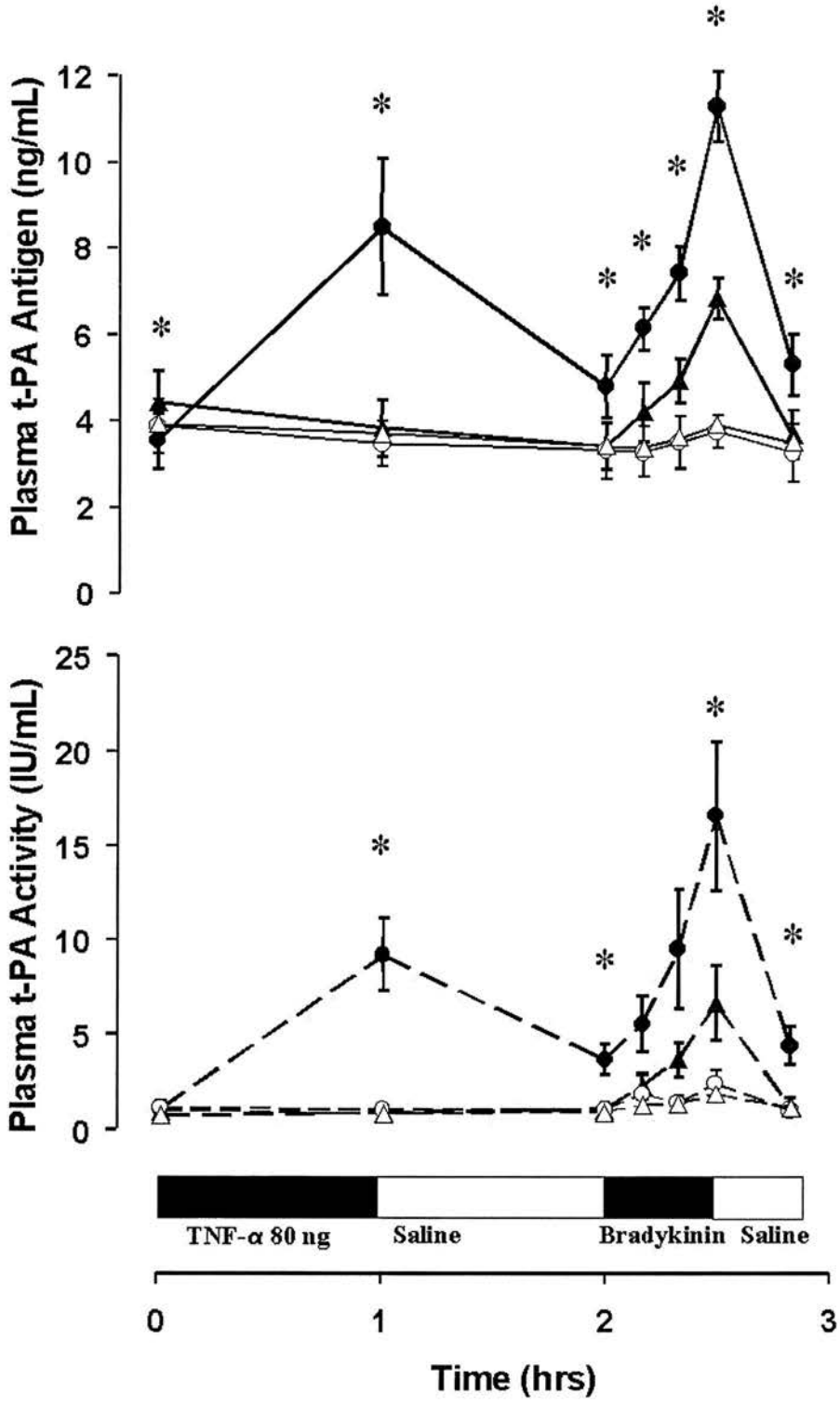


Figure 4.6 Protocol 2: Plasma concentrations of t-PA antigen (solid lines) and activity (dashed lines) in infused (closed symbols) and non-infused arm (open symbols) following 1 hour intra-arterial infusion of TNF-α (circles) or saline placebo (triangles) and during bradykinin infusion. $P<0.001$ (ANOVA) for all infused arm responses. $P<0.001$ (ANOVA, infused *vs* non-infused arm) for all responses. $P<0.001$ (ANOVA, TNF-α vs saline placebo) for infused arm responses. *$P<0.05$ (paired *t*-test; TNF-α vs placebo) for infused arm responses

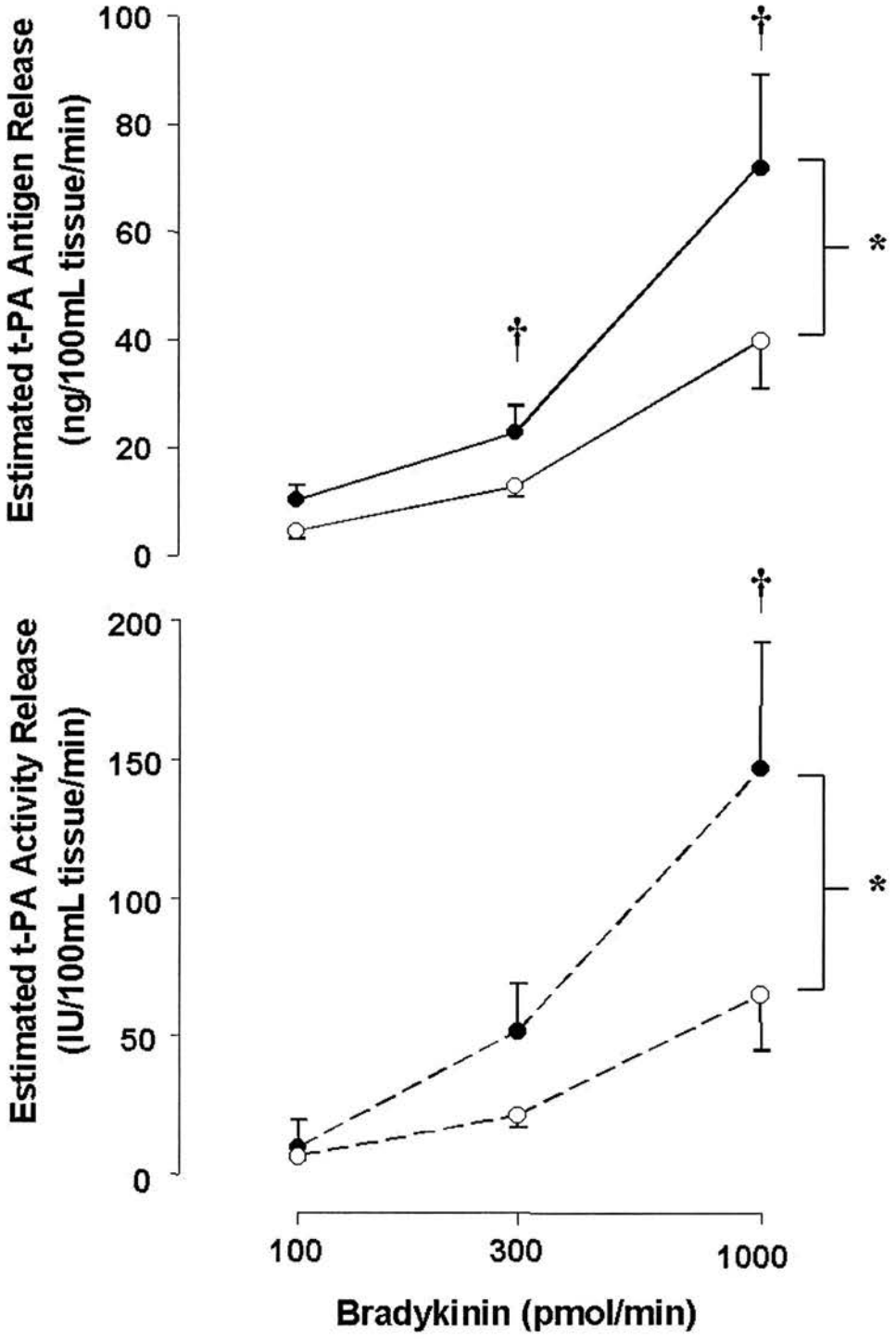


Figure 4.7 Protocol 2: Estimated net t-PA antigen (solid lines) and activity (dashed lines) release during bradykinin infusion corrected for baseline t-PA concentrations in subjects who were pre-treated with TNF-α (closed circles) or saline placebo (open circles).
$P<0.001$ (ANOVA) for all responses. *$P<0.05$ (ANOVA, TNF- α vs saline placebo).
†$P \leq 0.05$ (paired t-test; TNF-α vs saline placebo) .

4.5 Discussion

We have demonstrated for the first time that local intra-arterial TNF-α administration can be used to generate an *in vivo* local vascular inflammatory model in humans that is associated with endothelial dysfunction and a unique profile of endogenous t-PA release. In contrast to IL-6 and LPS, TNF-α causes a slow-onset, sustained and selective release of t-PA from the vascular endothelium in the absence of basal vasomotor effects. Moreover, pre-treatment with TNF-α attenuates endothelium-dependent vasodilatation, but augments acute bradykinin-induced t-PA release. These findings indicate that TNF-α and acute vascular inflammation have complex effects on endothelial function. Whilst the pro-fibrinolytic actions may reflect a protective mechanism in acute inflammation, TNF-α also directly impairs endothelium-dependent vasomotor responses.

4.5.1 Model of Local Vascular Inflammation

We have here developed a model of local vascular inflammation *in vivo* in man. Using unilateral intra-brachial infusions, we achieved high local plasma TNF-α concentrations that were comparable to the plasma concentrations seen in patients with severe heart failure [McMurray *et al* 1991]. A direct local vascular and endothelial inflammatory response was confirmed by the local rise in plasma IL-6 and t-PA concentrations. However, the fibrinolytic effects of TNF-α were not mediated through IL-6 release since isolated IL-6 infusion had no effect on t-PA release. Indeed, in pilot studies, we have found that high dose intra-brachial IL-6

infusion, sufficient to increase plasma IL-6 concentrations to >100 pg/mL, have also failed to produce significant effects on forearm blood flow or t-PA release.

4.5.2 Effects of TNF-α on t-PA Release

The profile of t-PA release during local intra-arterial TNF-α administration is unique and has not been previously described *in vivo* in man. Previous studies in healthy volunteers have reported changes in plasma fibrinolytic and coagulation factors during systemic TNF-α administration [van der Poll *et al* 1990, 1991]. TNF-α has pleiotropic effects and may cause these effects through actions on specific tissues or via secondary mediators released from organs such as the liver. In the present study, we have assessed local peripheral vascular responses to direct intra-arterial TNF-α and have shown that it causes selective endothelial t-PA release in the forearm without demonstrable effects on plasma von Willebrand factor or PAI-1 antigen concentrations. Although there was a modest rise in the IL-6 and t-PA concentrations in the non-infused arm, this may represent overspill from the infused arm where the concentrations increased by up to 20-fold. In contrast to previous systemic studies [van der Poll *et al* 1990, 1991], subjects here remained asymptomatic and there was no associated pyrexia consistent with the absence of a major systemic response.

We [Newby *et al* 1997a] and others [Brown *et al* 1999; Jern *et al* 1994] have previously reported acute rapid t-PA release during intra-arterial substance P, bradykinin and methacholine infusions. Using these agents, there is a near instantaneous onset and offset of action with no sustained increase in t-PA release after cessation of administration. Moreover, there is always an associated change in

vascular tone and regional blood flow since these agents also cause vasodilatation. In contrast, TNF-α administration had no effect on basal blood flow, and caused a slow onset and sustained release of t-PA that was not apparent until 20 min after commencement of the infusion and continued for at least 3 hours after its cessation.

Endothelial cells synthesise and secrete t-PA both constitutively and facultatively. The facultative release of t-PA occurs in response to stimulation by a number of physiological agonists including thrombin and bradykinin [Tranquille & Emeis 1989]. This large and rapid release arises from the translocation of a dynamic intracellular storage pool of t-PA [van den Eijnden-Schrauwen *et al* 1995; Tranquille & Emeis 1989]. Agonists, such as bradykinin, are likely to stimulate t-PA release via exocytosis of these granules because of the near instantaneous release of t-PA and the *ex vivo* animal evidence that inhibition of protein synthesis by cycloheximide has no effect on bradykinin-induced acute t-PA release [Tranquille & Emeis 1989]. The profile of t-PA release seen with TNF-α is distinct from this pathway. Although the mechanism has not been elucidated in our study, we speculate that it is likely to arise from an increase in *de novo* t-PA synthesis and its constitutive release rather than through the previously described facultative pathways. However, the initial detectable rise in t-PA release seen at 20 min may be too early for protein synthesis to occur. Other potential mechanisms such as activation of adherent leukocytes may cause the generation of secondary mediators that enhance t-PA release.

Infusion of TNF-α appears to cause an isolated increase in local t-PA release without affecting other endothelium-derived fibrinolytic or haemostatic factors: namely PAI-

1 and von Willebrand factor respectively. This suggests a selective and specific action of TNF-α on the endothelium and forearm vascular bed. The fall in plasma PAI-1 activity seen in the infused arm during high dose TNF-α infusion is likely to reflect the magnitude of the local t-PA released and its immediate binding with PAI-1. Although other workers have reported elevated plasma PAI-1 antigen concentrations within three hours of systemic administration of TNF-α and endotoxin [van der Poll *et al* 1991, 1997], we did not detect any changes in PAI-1 antigen concentrations. This suggests that cytokine-induced increases in PAI-1 release are mediated through a systemic mechanism, such as hepatic or adipocyte synthesis, or produced in response to markedly elevated systemic plasma t-PA concentrations.

4.5.3 Effects of TNF-α on Endothelium-dependent Vasomotion

Bhagat and Vallance have previously shown that TNF-α directly induced endothelial dysfunction in the venous circulation of healthy volunteers [Bhagat & Vallance 1997]. We have extended these findings and demonstrated that TNF-α also impairs resistance vessel endothelium-dependent vasodilatation, possibly through the development of acute arterial endothelial injury. The effects of TNF-α were specific for the endothelium since endothelium-independent vasodilatation to the nitric oxide donor, sodium nitroprusside, was unaltered.

TNF-α may alter endothelial vasomotor responses through various mechanisms including decreased constitutive nitric oxide synthase expression, increased inducible nitric oxide synthase expression, and enhanced production of reactive oxygen species

[Yoshizumi *et al* 1993; Matsubara *et al* 1986]. Further studies are needed to clarify the precise mechanism, although it is tempting to speculate that TNF-α may, in part, be responsible for inducing endothelial dysfunction in cardiovascular conditions associated with inflammation.

4.5.4 Effects of TNF-α on Acute Endogenous Fibrinolysis

We have further demonstrated that TNF-α potentiates bradykinin-induced endothelial t-PA release despite reduced endothelium-dependent vasodilatation. This suggests that acute local vascular inflammation induces anti-thrombotic properties that may represent an adaptive response to inhibit intravascular thrombus deposition at sites of vascular injury. In particular, molecular and pharmacological evidence supports the role of bradykinin B2 receptors in the acute phase of inflammation, and upregulation of B2 receptors by TNF-α may account for the observed pro-fibrinolytic effect. The augmented bradykinin response is also likely to reflect the proposed increase in *de novo* t-PA production induced by TNF-α pre-treatment.

4.5.5 Clinical Implications

There is a consistent link between endothelial dysfunction and cardiovascular disease, with impaired endothelium-dependent vasodilatation having been described in atherosclerotic conditions [Zeiher *et al* 1991] and its associated risk factors, such as hypercholesterolaemia [Chowienczyk *et al* 1992]. The major findings of our study will be particularly pertinent to cardiovascular conditions, such as acute coronary syndromes and congestive heart failure, in which inflammation and impaired endothelium-dependent vasodilatation occur.

We have found that while TNF-α adversely affects certain aspects of endothelial function, such as endothelium-dependent vasodilatation, it enhances other protective mechanisms, such as the endogenous fibrinolytic capacity. This reflects the complex and pleiotropic nature of TNF-α which functions as part of the normal host surveillance mechanisms and responses to tissue injury. These diverse effects may explain some of the contradictory findings of recent clinical studies. For example, in patients with heart failure, TNF-α antagonism causes marked improvements in endothelium-dependent vasodilatation [Fichtlscherer *et al* 2001] but has failed to demonstrate clinical benefit in the RECOVER and RENAISSANCE randomised controlled trials [Louis *et al* 2001]. Thus, the benefits of restoring endothelium-dependent vasomotor function by TNF-α antagonism may be counterbalanced by inhibiting other potentially beneficial effects such as enhancing acute endogenous t-PA release.

4.5.6 Conclusions

This is the first study to delineate the direct effects of intra-arterial TNF-α administration on local vascular tone and endogenous fibrinolysis *in vivo* in man. It supports the crucial role of TNF-α in cardiovascular disease and provides evidence for its direct and pleiotropic effects on the circulation and endogenous fibrinolysis. Our findings have particular implications for the future development of effective anti-cytokine and anti-inflammatory strategies in cardiovascular disease.

Chapter 5

Endothelial Dysfunction In Patients With Recent Myocardial Infarction And Hyperhomocysteinaemia

Effects of Vitamin Supplementation

Chia S, Wilson R, Ludlam CA, Webb DJ, Flapan AD, Newby DE.

Endothelial Dysfunction In Patients With Recent Myocardial Infarction And Hyperhomocysteinemia. *Effects of Vitamin Supplementation.*

Clinical Science (Lond). 2005;**108**:65-72

5.1 Summary

Hyperhomocysteinaemia is a pro-thrombotic condition that may cause oxidative endothelial injury and impair endogenous fibrinolysis. Vitamin supplementation enhances endothelial function in hyperhomocysteinaemic patients, but responses in patients with co-existing coronary artery disease have been variable. It is unknown whether hyperhomocysteinaemia is associated with reduced fibrinolytic responses in patients with coronary artery disease. The study aims were to test the hypothesis that patients with recent myocardial infarction and hyperhomocysteinaemia have impaired endothelium-dependent vasomotion and fibrinolysis that is rectified by vitamin supplementation. From a cohort of one hundred and twenty patients admitted with acute myocardial infarction, eighteen patients were recruited from the upper (n=9) and lower (n=9) plasma homocysteine quartiles into a randomised double-blind placebo-controlled crossover trial. Following a 4-week course of placebo or folate/cyanocobalamin/pyridoxine supplements, forearm blood flow (FBF) was measured using venous occlusion plethysmography during intra-arterial substance P (4-16 pmol/min), acetylcholine (5-20 μg/min) and sodium nitroprusside (2-8 μg/min) infusions. All vasodilators caused dose-dependent increases in infused FBF (P<0.05). Patients in the upper homocysteine quartile (16.8±2.9 *vs* 7.9±0.7 μmol/L; P=0.003) had reduced vasodilatation to acetylcholine (P=0.01) and substance P (P<0.05) but not sodium nitroprusside. There were no differences in substance P-induced tissue plasminogen activator (t-PA) release. Vitamin supplementation increased serum folate and vitamin B_{12} concentrations (P<0.05) but

did not significantly lower homocysteine, or affect FBF or fibrinolytic responses. In patients with recent myocardial infarction, we conclude that hyperhomocysteinaemia is associated with impaired endothelium-dependent vasodilatation but no alteration in the acute fibrinolytic capacity. This endothelial vasomotor dysfunction is unaltered by vitamin supplementation.

5.2 Introduction

Several prospective and case-control studies have shown that elevated plasma homocysteine concentrations are an independent risk factor for the development of atherothrombotic vascular disease as well as a prognostic marker in ischaemic heart disease [Clarke *et al* 1991; Stampfer *et al* 1992; Welch & Loscalzo 1998]. Plasma homocysteine concentrations are consistently higher in patients with premature peripheral and cerebrovascular diseases [Boers *et al* 1985], and almost a third of patients with premature coronary artery disease are found to have hyperhomocysteinaemia [Clarke *et al* 1991]. In addition, apparently healthy men with plasma homocysteine concentrations 12% above the upper limit of normal have a threefold increased risk of acute myocardial infarction [Stampfer *et al* 1992], and in patients with ischaemic heart disease there is an increased mortality associated with plasma concentrations greater than 9 μmol/L [Nygard *et al* 1997].

The vascular endothelium plays a central role in the control of blood flow, haemostasis and endogenous fibrinolysis [Vane *et al* 1990], and endothelial dysfunction independently predicts cardiovascular events [Heitzer *et al* 2001; Halcox *et al* 2002]. Although the mechanism of vascular damage is unclear, homocysteine may promote atherogenesis through oxidative endothelial injury that is mediated by cytotoxic reactive oxygen species [Stamler *et al* 1993; Lentz *et al* 1996; Loscalzo 1996; Kanani *et al* 1999]. Indeed, acute and chronic hyperhomocysteinaemia are associated with impaired endothelium-dependent flow-mediated dilatation of the brachial artery [Celermajer *et al* 1993a; Tawakol *et al* 1997; Kanani *et al* 1999].

Hyperhomocysteinaemia is a prothrombotic state [Welch & Loscalzo 1998]. We [Labinjoh *et al* 2001] and others [Freyburger *et al* 1997] have previously shown that hyperhomocysteinaemia induced by oral methionine loading is associated with alterations in endogenous fibrinolysis in healthy subjects and patients with premature vascular disease. However, the influence of chronic hyperhomocysteinaemia on the acute fibrinolytic capacity is unknown and is the subject of debate [Kuller & Evans 1998]. Interestingly, there is an association between plasma homocysteine and t-PA antigen concentrations in stroke patients [Lindgren *et al* 1996].

Homocysteine is formed by the demethylation of methionine and is an intermediate in the formation of cystathionine and cysteine (Figure 5.1). The trans-sulphuration of homocysteine to cysteine is dependent upon pyridoxine (vitamin B_6), and the demethylation of methionine upon cobalamin (vitamin B_{12}) and folate (Ueland & Refsum 1989; Kang *et al* 1992). Vitamin supplementation with folic acid, vitamin B_6 and vitamin B_{12}, is safe and may reduce plasma homocysteine concentrations [Homocysteine Lowering Trialists' Collaboration 1998]. Whilst endothelial function is enhanced following treatment with folic acid in patients with hyperhomocysteinaemia [Woo *et al* 1999, 2002] and hypercholesterolaemia [Verhaar *et al* 1999], the response in patients with coronary artery disease has been variable [Chambers *et al* 2000; Title *et al* 2000; Thambyrajah *et al* 2001; van Dijk *et al* 2001; Doshi *et al* 2002]. Furthermore, it is not known whether elevated plasma homocysteine concentrations are associated with reduced resistance vessel vasomotor responses in patients with established coronary artery disease.

The aim of this study was to test the hypotheses that,

(1) In patients with recent myocardial infarction, elevated plasma homocysteine concentrations are associated with impaired endothelium-dependent vasodilatation and endogenous fibrinolytic capacity, and

(2) Vitamin supplementation (with folic acid, vitamin B_6 and vitamin B_{12}) would both lower plasma homocysteine and restore endothelial function.

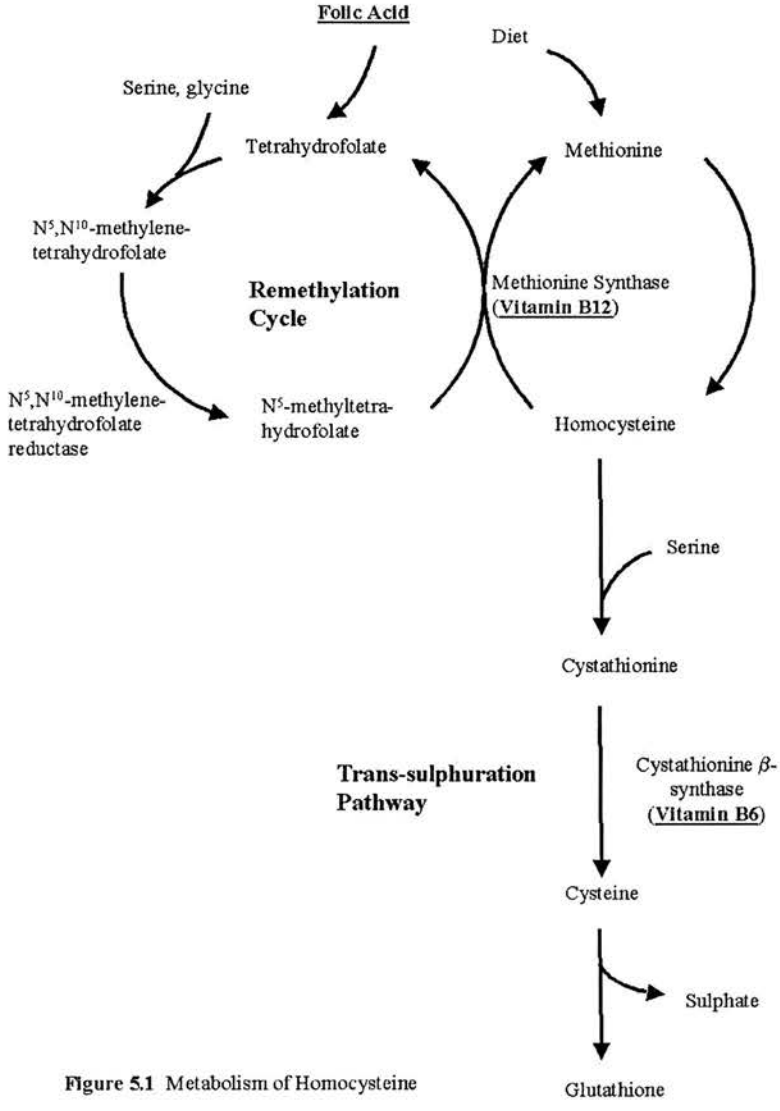


Figure 5.1 Metabolism of Homocysteine

5.3 Methods

5.3.1 Subjects

One hundred and twenty patients admitted with an acute myocardial infarction were recruited into the trial. Myocardial infarction was defined as typical ischaemic cardiac pain associated with elevation of cardiac markers (greater than twice the upper limit of normal) and electrocardiographic evidence of myocardial ischaemia. Exclusion criteria were atrial fibrillation on warfarin therapy, impaired renal function (serum creatinine >120 μmol/L), diabetes mellitus, requirement for folic acid supplementation or pernicious anaemia. The written informed consent of each subject was obtained before entry into the study. All studies were undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki (1996).

5.3.2 Drugs

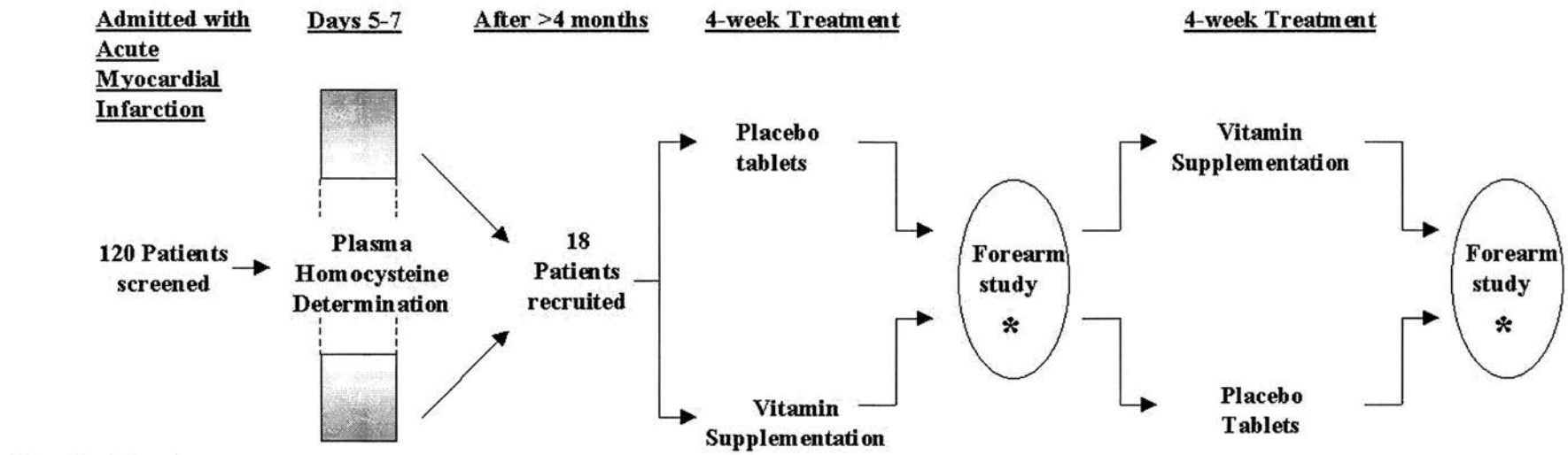
Sucrose placebo tablets (Thornton & Ross Ltd, Huddersfield, Untied Kingdom) or vitamin supplementation was given as a once daily oral preparation. Vitamin supplementation was administered as a combination therapy of folic acid (5 mg; Alpharma, Barnstaple, United Kingdom), vitamin B_6 (pyridoxine 10 mg; B R Pharma Ltd., Barnet, Untied Kingdom) and vitamin B_{12} (cyanocobalamin 100 μg; Goldshield Pharmaceuticals Ltd., Croydon, Untied Kingdom).

Pharmaceutical-grade substance P (Clinalfa, Läufelfingen, Switzerland), acetylcholine (Cibavision Ophthalmics, Southampton, UK) and sodium nitroprusside

(David Bull Laboratories, Faulding, UK) were administered as intra-arterial infusions following dissolution in saline. All solutions were freshly prepared on the day of study.

5.3.3 Study Design (Figure 5.2)

Fasting plasma homocysteine concentrations were determined in all patients on days 5-7 following acute myocardial infarction [Egerton *et al* 1996]. From the upper and lower plasma homocysteine concentration quartiles, 9 patients in each quartile were recruited into a randomised, double blind, balanced-block, placebo-controlled crossover trial at least 4 months after the index event. All patients received 2 separate 4-week courses of oral sucrose placebo or vitamin supplementation, and attended at the end of each 4-week treatment period. On each study day, medications were withheld and subjects attended after a 4-hour fast and rested recumbent in a quiet, temperature-controlled room. Strain gauges and cuffs were applied, and the brachial artery of the non-dominant arm was cannulated. Venous cannulae were inserted into large subcutaneous veins of both arms. After 30 min equilibration with saline infusion, intra-arterial substance P (4-16 pmol/min), acetylcholine (5-20 μg/min) and sodium nitroprusside (2-8 μg/min) were administered in a randomised order for 6-10 min at each dose and separated by 20 min washout periods [Newby *et al* 1997a, 1998a]. Venous samples were taken at baseline for determination of plasma homocysteine and serum folate and vitamin B_{12} concentrations (as described in Chapter 2.4.2.3), and during substance P infusion for fibrinolytic factor assays (as described in Chapter 2.4.2.1).



Study Design

* Forearm Study Protocol

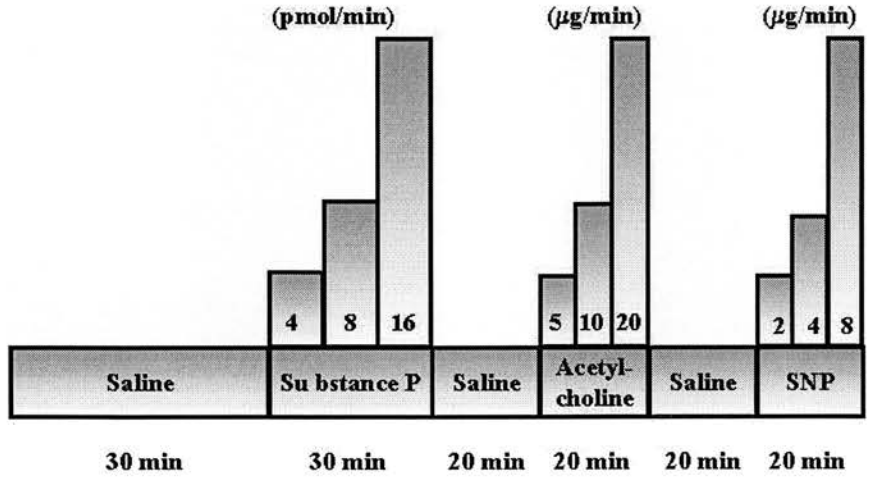


Figure 5.2
Study Design (upper panel) and forearm study protocol for intra-arterial drug infusion (lower panel).

5.3.4 Data Analysis and Statistics

Estimated net t-PA antigen and activity release has been defined previously in Chapter 2.5.2. Data were examined, where appropriate, by analysis of variance (ANOVA) with repeated measures and two-tailed paired Student's *t*-test. All results are expressed as mean (SEM) or mean (95% Confidence Interval, CI). Statistical significance was assigned at the 5% level.

5.4 Results

5.4.1 Baseline and Biochemical Characteristics

From the upper and lower plasma homocysteine quartiles, 18 patients (9 patients in each quartile) were recruited into the randomised controlled trial. Apart from plasma homocysteine concentrations ($P<0.01$, *t*-test), which were significantly different by design, there were no significant differences between the baseline clinical characteristics or medical therapies in the two groups of patients (Tables 5.1 and 5.2).

All subjects tolerated placebo and vitamin supplementation and no side effects were reported or noted. Serum folate and vitamin B_{12} concentrations were increased following vitamin supplementation compared to placebo in both patients groups (Table 5.2; $P<0.001$ and $P<0.05$ respectively, *t*-test), and plasma homocysteine concentrations appeared to be reduced by $\approx$16% with active treatment in hyperhomocysteinaemic patients, but this was not statistically significant ($P=0.3$, *t*-test). Vitamin supplementation had no significant effects on heart rate, blood pressure or basal forearm blood flow in either patient group.

Table 5.1 Patients Characteristics

	Patients in Upper Quartile (n=9)	**Patients in Lower Quartile (n=9)**
Age, years	54±2	57±5
Sex (male:female)	9:0	8:1
Mean Arterial Pressure, mmHg	88±5	86±2
Heart rate, bpm	59±2	54±2
Type of Myocardial Infarction		
Anterior, (%)	2 (22)	3 (33)
Inferior, (%)	6 (67)	3 (33)
Other, (%)	1 (11)	3 (33)
Peak Creatine Kinase, U/L	790±230	1900±550
Received thrombolysis, (%)	6 (67)	6 (67)
Medication		
Anti-platelet therapy, (%)	9 (100)	9 (100)
β-adrenoceptor antagonists, (%)	8 (89)	6 (67)
ACE inhibitors, (%)	5 (56)	5 (56)
HMG CoA reductase inhibitors, (%)	8 (89)	7 (77)
Risk Factors		
Smokers, (%)	6 (67)	3 (33)
History of hypertension, (%)	3 (33)	2 (22)
Hypercholesterolemia, (%)	8 (89)	7 (77)
Diabetes Mellitus, (%)	0 (0)	0 (0)

Mean±SEM

Table 5.2

Plasma homocysteine, serum folate and vitamin B_{12} concentrations following vitamin supplementation

	Upper Quartile		***Lower Quartile***	
	Placebo	**Treatment**	**Placebo**	**Treatment**
Plasma Homocysteine, μmol/L	16.8±2.8*	14.2±1.1*	7.9±0.7	7.8±0.7
Serum Folate, μg/L‡	8.6±1.8	17.7±1.6†	9.0±1.2	19.9±0.1†
Serum Vitamin B_{12}, ng/L	462±136	580±149†	380±39	431±48†

Mean±SEM

*$P<0.01$ Upper *vs* Lower Quartile, *t*-test

†$P<0.05$ Vitamin *vs* placebo, *t*-test

‡ Upper limit of serum folate assay was 20 μg/L - 13 patients had concentrations above this after supplementation and were taken as 20 μg/L.

5.4.2 Endothelium-dependent Vasomotion

Substance P, acetylcholine and sodium nitroprusside caused dose-dependent increases in blood flow of the infused forearm in both patient groups on each study day (Figure 5.3; $P<0.05$, ANOVA). In comparison to patients in the lower quartile, hyperhomocysteinaemic patients had significantly reduced forearm blood flow responses to acetylcholine and substance P (Figure 5.3; $P=0.01$ and $P<0.05$ respectively, ANOVA), but there were no significant differences in the blood flow responses to sodium nitroprusside (P=NS, ANOVA).

Neither endothelium-dependent nor -independent vasodilatation were significantly influenced by vitamin treatment in either patient group (P=NS for all, ANOVA). For the hyperhomocysteinaemic group, the mean difference for the response to vitamin treatment at the peak dose was 1.1 mL/100 mL/min (95% CI, -0.8 to +2.9) for substance P, 0.3 mL/100 mL/min (95% CI, -1.3 to +1.8) for acetylcholine, and –0.3 mL/100 mL/min (95% CI, -2.8 to +2.1) for sodium nitroprusside. For the lower quartile group, the mean difference for the response to vitamin treatment at the peak dose was 2.0 mL/100 mL/min (95% CI, -0.6 to +4.6) for substance P, -0.8 mL/100 mL/min (95% CI, -2.9 to +1.2) for acetylcholine, and 1.7 mL/100 mL/min (95% CI, -2.8 to +6.2) for sodium nitroprusside.

5.4.3 Fibrinolytic Responses

Baseline plasma t-PA and PAI-1 antigen, and t-PA activity concentrations were similar in both groups (Tables 5.3 and 5.4). Substance P caused dose-dependent increases in plasma t-PA antigen and activity concentrations in the infused forearm

of all patients (Tables 5.3 and 5.4; $P<0.05$ for both, ANOVA). The responses were similar in both patient groups and were not influenced by vitamin supplementation (P=NS, ANOVA). The mean difference of t-PA antigen release for the response to vitamin supplementation at the peak substance P dose was –2.7 ng/100 mL/min (95% CI, -15.5 to +10.2, P=NS, t-test) in the upper quartile group and –7.4 ng/100 mL/min (95% CI, -24.3 to +9.5, P=NS, t-test) in the lower quartile group. Plasma PAI-1 concentrations were unaffected by substance P infusion or vitamin treatment (P=NS, t-test).

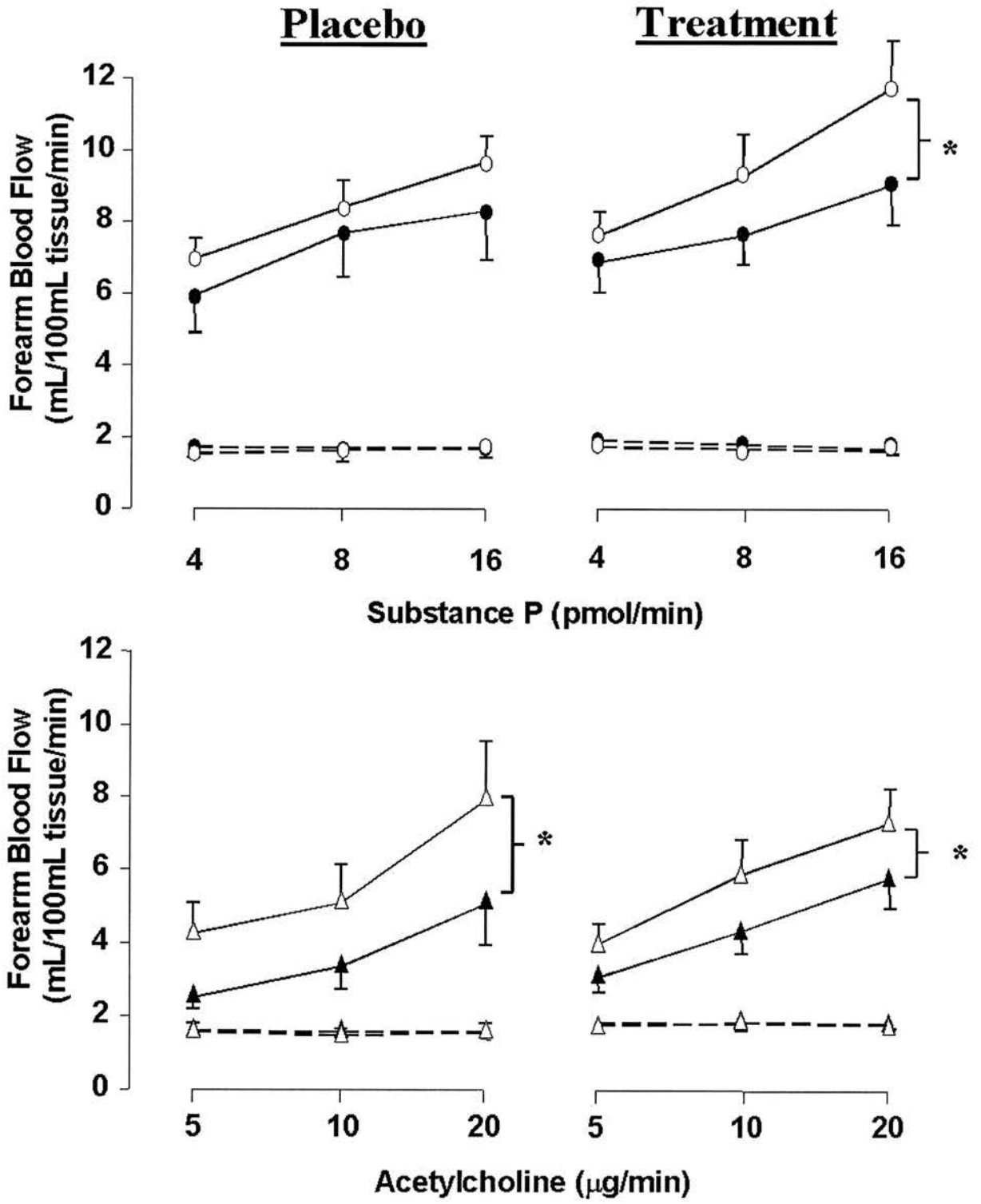


Figure 5.3 Infused (solid line) and non-infused (dashed line) forearm blood flow responses to incremental doses of substance P (upper panel) and acetylcholine (lower panel) in patients in upper (closed symbols) and lower quartiles (open symbols) following placebo (left panel) and vitamin supplementation (right panel). $P<0.05$ (ANOVA, for all infused arm responses); *$P<0.05$ (ANOVA, upper *vs* lower quartile).

Table 5.3
Plasma tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) antigen concentrations and release during substance P infusion

		Upper Quartile				Lower Quartile			
	Substance P (pmol/min)	**Placebo**		**Treatment**		**Placebo**		**Treatment**	
		Infused arm	**Non-infused arm**	**Infused arm**	**Non-infusec arm**	**Infused arm**	**Non-infused arm**	**Infused arm**	**Non-infused arm**
t-PA antigen (ng/ml)	**Baseline**	7.7±1.2	8.6±1.4	7.5±1.0	8.2±1.4	6.6±1.2	6.5±1.3	7.2±1.3	7.6±1.3
	4	9.8±1.5	8.3±1.3	9.2±1.0	7.7±1.3	7.6±0.9	7.0±1.1	8.1±1.4	7.6±1.4
	8	9.4±1.5	8.1±1.1	10.3±1.1	7.8±1.0	9.3±1.4	7.1±1.2	8.9±1.5	7.6±1.5
	16	12.7±2.4*	7.8±0.9	11.1±1.4*	7.6±1.1	10.6±1.2*	6.7±1.3	10.5±1.6	7.9±1.4
Estimated t-PA antigen release (ng/100 mL/min)	**Baseline**	-1.1±0.7		-1.2±1.1		0.4±0.7		-0.3±0.4	
	4	4.0±3.2		5.4±3.4		3.4±2.2		1.4±1.5	
	8	4.7±4.1		11.1±4.2		9.8±3.7		5.9±2.4	
	16	21.6±6.7*		18.9±5.2*		24.3±8.4*		15.6±3.9*	
Net t-PA antigen release (AUC)		18.9±9.3		25.4±9.0		24.2±8.5		14.9±4.2	
PAI-1 antigen (ng/mL)	**Baseline**	47±5	48±4	44±4	48±5	45±14	46±14	44±11	46±13
	16	48±4	54±3	45±4	50±5	44±15	44±15	46±12	49±15

AUC = Area under curve Mean ± SEM
*$P<0.05$ for all responses during substance P infusion, ANOVA

Table 5.4 Plasma tissue plasminogen activator (t-PA) activity concentrations and release during substance P infusion

		Upper Quartile				Lower Quartile			
	Substance P	**Placebo**		**Treatment**		**Placebo**		**Treatment**	
	(pmol/min)	**Infused arm**	**Non-infused arm**	**Infused arm**	**Non-infusec arm**	**Infused arm**	**Non-infused arm**	**Infused arm**	**Non-infused arm**
t-PA activity (IU/mL)	**Baseline**	0.9±0.2	0.8±0.2	0.5±0.1	0.6±0.1	1.0±0.3	1.0±0.3	0.8±0.2	0.8±0.3
	4	1.7±0.6	0.7±0.2	1.5±0.3	0.8±0.1	2.5±0.8	1.2±0.4	1.5±0.6	0.9±0.2
	8	1.9±0.7	0.8±0.3	2.3±0.4	0.7±0.2	2.8±0.9	1.2±0.4	2.0±0.9	0.9±0.2
	16	3.1±0.8*	1.0±0.2	2.9±0.5*	0.8±0.2	3.9±1.1*	1.5±0.4	2.7±1.0*	0.9±0.2
Estimated t-PA activity release (IU/100 mL/min)	**Baseline**	0.0±0.1		-0.1±0.1		-0.0±0.1		0.1±0.2	
	4	2.5±1.4		3.1±1.3		6.0±2.1		2.2±1.2	
	8	3.6±1.6		7.0±1.7		8.4±3.6		4.6±2.1	
	16	9.3±2.9*		11.7±3.6*		16.4±6.9*		10.2±3.4*	
Net t-PA activity release (AUC)		10.7±4.2		15.9±4.7		21.6±8.7		12.0±4.9	

AUC = Area under curve
Mean ± SEM
*$P<0.05$ for all responses during substance P infusion, ANOVA

5.5 Discussion

We have demonstrated that, in patients with a recent myocardial infarction, elevated plasma homocysteine concentrations is associated with impaired endothelium-dependent vasodilatation without affecting acute endogenous t-PA release. However, vitamin supplementation failed to reduce plasma homocysteine concentrations or improve endothelial vasomotor function.

5.5.1 Endothelium-dependent Vasodilatation

Recent studies have shown that homocysteine is associated with endothelial dysfunction. Lentz *et al* [Lentz *et al* 1996] reported that in monkeys with diet-induced hyperhomocysteinaemia, endothelium-dependent vasodilatation is impaired in carotid artery rings *in vitro* and hindlimb resistance vessels *in vivo*. Celermajer *et al* [Celermajer *et al* 1993a] documented abnormal endothelium-dependent vasodilatation in children with severe hyperhomocysteinaemia due to homozygous homocystinuria. Impaired endothelium-dependent vasodilatation is detected in healthy subjects with acute hyperhomocysteinaemia induced by oral methionine loading [Bellamy *et al* 1998; Kanani *et al* 1999], as well as in patients with chronic hyperhomocysteinaemia who are free from clinical manifestations of atherosclerotic disease [Tawakol *et al* 1997; Woo *et al* 1999]. In this study, we have extended these findings to patients with recent myocardial infarction and demonstrated impaired vasomotor responses in those with elevated plasma homocysteine concentrations.

The vascular endothelium plays a critical role in the control of vascular homeostasis by regulating vascular tone, platelet activity, coagulation and fibrinolysis [Vane *et al* 1990], and endothelial dysfunction is believed to be an early step in the pathogenesis and pathophysiology of atherosclerosis. Although patients with coronary artery disease typically demonstrate endothelial dysfunction, there is considerable heterogeneity in the magnitude of impairment in individuals with similar risk factor profiles. This is of particular interest because the extent of coronary as well as peripheral endothelial dysfunction independently predicts the long-term risk of acute cardiovascular events, including sudden cardiac death, myocardial infarction and revascularisation procedures [Heitzer *et al* 2001; Halcox *et al* 2002]. Recent prospective data have indicated that in patients with established coronary artery disease, homocysteine is a significant predictor of mortality independent of other traditional risk factors [Nygard *et al* 1997; Anderson *et al* 2000]. Our findings therefore support the role of homocysteine as a secondary risk marker, suggesting that this may be mediated through its effects on endothelial function.

5.5.2 Endogenous Fibrinolysis

Although homocysteine impairs endothelial vasomotor function, it does not appear to have a major effect on endothelium-dependent fibrinolytic capacity, as both basal and stimulated release of t-PA or PAI-1 were not significantly different between the two patient groups. Hyperhomocysteinaemia is a prothrombotic condition and may interfere with the anti-thrombotic and fibrinolytic mechanisms of the endothelium and alter endothelial protein secretory pathways. Although endothelial cell-associated t-PA activity is reduced in homocysteine-treated cells [Hajjar 1993], our

present study failed to detect reduced fibrinolytic activity *in vivo*. This is consistent with data indicating that homocysteine might perturb the intrinsic fibrinolytic potential by reducing the functional binding site for t-PA without altering the catalytic capability of t-PA or affecting t-PA synthesis and secretion [Hajjar 1993].

5.5.3 Effects of Vitamin Supplementation on Plasma Homocysteine

Previous studies have shown that treatment with folic acid and B vitamins can lower plasma homocysteine concentrations to varying degree. The Homocysteine Lowering Trialists' meta-analysis predicted a 20-30% reduction in homocysteine in patients with plasma concentrations above 12 μmol/L taking folic acid, and a further small additional effect with vitamin B_{12} but not B_6 [Homocysteine Lowering Trialists' Collaboration 1998]. However, a more modest 11-14% reduction is seen in patients with coronary artery disease who consumed fortified breakfast cereals [Malinow *et al* 1998; Bostom *et al* 2002]. The limited homocysteine lowering seen in our study was probably related to the relatively mild hyperhomocysteinaemia and normal folate concentrations in our study population as well as the confounding effects of dietary folate fortification.

5.5.4 Effects of Vitamin Supplementation on Endothelial Responses

We did not detect an improvement in endothelium-dependent vasodilatation or endogenous fibrinolytic capacity following vitamin supplementation in this present study. Although this may not be surprising in the absence of significant homocysteine reduction, Doshi *et al* [Doshi *et al* 2002] have suggested that the acute effects of folic acid on endothelial function may occur by a mechanism independent

of homocysteine lowering. Moreover, the evidence on the beneficial effects of vitamin supplementation on endothelial function is conflicting. Chambers *et al* [Chambers *et al* 2000] and Title *et al* [Title *et al* 2000] demonstrated improved endothelial function in patients with coronary artery disease following folic acid treatment with or without vitamin B_{12}. However, our findings are consistent with those of other investigators who failed to detect improved endothelial function in a similar patient population, in healthy siblings of patients with premature atherothrombotic disease or in patients with renal impairment [Thambyrajah *et al* 2000, 2001; van Dijk *et al* 2001]. These contradictory findings may be related in part to the presence of other cardiovascular risk factors such as hypertension or hypercholesterolaemia, which may contribute to endothelial injury [Panza *et al* 1990; Chowienczyk *et al* 1992] but would not be expected to respond to folic acid or B vitamins. The vascular endothelium in patients with established coronary artery disease may have also been subjected to chronic injury and therefore less responsive to intervention. Furthermore, while the above studies adopted flow-mediated dilatation as a non-invasive method of assessing conduit artery endothelial function, in this and other studies, we have focussed on the function of endothelium within resistance vessels [Newby *et al* 1997a, 2001]. Conduit artery and microvascular endothelial cells have distinct phenotypic differences, and responses to mechanical rather than pharmacological stimuli may also differ, and may contribute to the apparent disparity in the responses.

There is further controversy regarding the effects of vitamin supplementation on cardiovascular outcomes in patients undergoing percutaneous coronary intervention.

Contrary to earlier reports that vitamin supplementation may reduce the rate of restenosis and adverse outcomes following coronary artery angioplasty [Schnyder *et al* 2001, 2002], Lange *et al* [Lange *et al* 2004] have recently demonstrated that folate therapy following coronary stenting may increase the risk of in-stent restenosis. The underlying mechanism for these findings remains uncertain, but it is possible that the proliferative effects of folic acid may promote the growth of neointimal cells within implanted stents. Therefore more prospective data are needed before any recommendations can be made at all regarding the use of vitamin supplementation in coronary artery disease.

5.5.5 Study Limitations

There are several potential limitations to our study. First, we studied peripheral vascular function and thus these findings may not be directly applicable to other vascular beds. However, endothelial dysfunction is often a generalised process, and we have previously shown consistent vasomotor and endogenous fibrinolytic responses between the forearm and coronary circulation [Newby *et al* 1999, 2001]. Second, the failure to improve endothelial function may be related to inadequate treatment duration, although studies have suggested that a 4-6 week treatment with folic acid can improve endothelial function [Verhaar *et al* 1999; Doshi *et al* 2002]. Third, the size of the study was small, and although it was powered to detect a 15-20% difference in t-PA release or forearm vasodilatation, it is possible that a smaller effect may have been missed. Fourth, we had used a placebo-controlled crossover design and there was the possibility of a carry-over effect of the vitamin therapy on endothelial function during placebo administration, despite a significant difference in

the serum concentrations of folate and vitamin B_{12}. Finally, we cannot rule out that homocysteine may be a marker of vascular injury rather than a mediator of endothelial dysfunction, although experimental data support the direct role of homocysteine in causing endothelial damage [Harker *et al* 1976; Hajjar 1993; Lentz *et al* 1996].

5.5.6 Conclusion

We have demonstrated that endothelium-dependent vasodilatation but not endogenous fibrinolysis is impaired in patients with recent myocardial infarction and elevated plasma homocysteine, and that this endothelial vasomotor dysfunction is not rectified by vitamin supplementation. These results provide further evidence for the role of homocysteine in vascular damage, but do not support the hypothesis that vitamin supplementation improves endothelial function in patients with established coronary artery disease.

Chapter 6

Preserved Endothelial Vasomotion And Fibrinolytic Function In Patients With Acute Stent Thrombosis Or In-stent Restenosis

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Preserved Endothelial Vasomotion And Fibrinolytic Function In Patients With Acute Stent Thrombosis Or In-stent Restenosis.

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6.1 Summary

Acute stent thrombosis and in-stent restenosis are serious complications of percutaneous coronary intervention (PCI) and may be associated with vascular or platelet abnormalities. We aimed to assess endothelium-dependent vasomotion, endogenous fibrinolysis and platelet function in patients with acute stent thrombosis or in-stent restenosis. Thirty-six subjects were enrolled into four groups: acute stent thrombosis, in-stent restenosis, uncomplicated PCI with stent implantation, and healthy matched controls. Forearm blood flow was measured using bilateral venous occlusion plethysmography during intra-brachial acetylcholine, substance P and sodium nitroprusside infusion. Venous blood samples were withdrawn for estimation of plasma fibrinolytic variables and platelet aggregometry. Acetylcholine, substance P and sodium nitroprusside caused dose-dependent increases in blood flow ($P<0.001$), and substance P caused a dose-dependent increase in tissue-type plasminogen activator (t-PA) release ($P<0.001$) in all groups. Thrombin, collagen, adenosine diphosphate and the thromboxane A2 analogue, U46619, caused dose-dependent platelet aggregation ($P<0.001$) in all groups. There were no significant between group differences in these responses except that, in keeping with aspirin therapy, collagen induced platelet aggregation was impaired in patient groups compared with healthy controls ($P<0.01$). Post-hoc analysis demonstrated a significant impairment of acute t-PA release in current smokers compared to non-smokers ($P<0.05$). Despite previous reports suggesting impaired vascular function, we conclude that endothelium-dependent vasomotion, endogenous fibrinolysis and platelet aggregation do not appear to play a major role in the pathogenesis of acute stent thrombosis or in-stent restenosis.

6.2 Introduction

Coronary artery stent implantation is a valuable adjunct to percutaneous transluminal coronary angioplasty. It reduces the absolute incidence of restenosis compared with balloon angioplasty by 10% to 15% and improves 6-month event free survival by 10% to 20% [Fischman *et al* 1994; Serruys *et al* 1994]. However, there is a small but significant risk of acute coronary stent thrombosis [Cutlip *et al* 2001] and in-stent restenosis that can have devastating consequences including myocardial infarction and death [Albiero *et al* 1997]. Whilst procedure-related complications such as persistent dissection, longer stent length and final minimal lumen diameter may be implicated in some cases of stent thrombosis or restenosis [Cutlip *et al* 2001], no underlying precipitant can be identified in many patients.

Stent thrombus formation is principally initiated by platelet aggregation which, in the absence of effective endothelium-derived vasoregulation and fibrinolysis, is then stabilised by the deposition of a fibrin mesh. The chronology of in-stent restenosis has been described as early thrombosis, followed by thrombus endothelialisation and infiltration by lymphocytes and monocytes, and finally smooth muscle cell migration and proliferation within the resolving thrombus [Schwartz *et al* 1993; Miller *et al* 1996; Komatsu *et al* 1998]. The initiation, propagation and stabilisation of acute stent thrombosis and in-stent restenosis are therefore dependent on several components: platelet aggregation, endothelial function, coagulation and fibrinolysis.

The purpose of the present study was to identify potential factors that may be implicated in the predisposition to acute stent thrombosis or in-stent restenosis using a case-control methodology. We assessed endothelium-dependent vasomotion, platelet aggregation and the acute endogenous fibrinolytic capacity in patients who have developed thrombotic complications following PCI and stent implantation.

6.3 Methods

6.3.1 Subjects

Twenty-six patients who had undergone PCI with stent implantation at least 6 months previously and ten age-and sex-matched healthy control subjects were recruited into the study. In the patient group, sixteen had developed complications of acute stent thrombosis within 48 hours of stent implantation (n=6) or in-stent restenosis within six months of intervention (n=10), and ten had no clinical evidence of stent thrombosis or restenosis at least one year following the procedure. Patients with procedural complications or sub-optimal stent insertion were excluded. All patients received thieopyridine therapy for 4 weeks after undergoing PCI, and none received a glycoprotein IIb/IIIa receptor antagonist during the procedure.

The investigation was performed with the written informed consent of each subject and the approval of the local research ethics committee, and conformed to the principles outlined in the Declaration of Helsinki.

6.3.2 Drugs

Pharmaceutical-grade substance P (Clinalfa, Läufelfingen, Switzerland), acetylcholine (Cibavision Ophthalmics, Southampton, United Kingdom) and sodium nitroprusside (David Bull Laboratories, Faulding, United Kingdom) were administered following dissolution in 0.9% saline. All solutions were freshly prepared on the day of study.

6.3.3 Study Design

Subjects attended on a single day following a 4-hour fast and then rested recumbent throughout the study. Venous blood was collected at baseline for platelet aggregometry and thrombophilia screen (as described in Chapters 2.4.2.5 and 2.4.3). Strain gauges and cuffs were applied, and the brachial artery of the non-dominant arm was cannulated under local anaesthesia. Venous cannulae were inserted into large subcutaneous veins of both arms. After 30 min equilibration with saline infusion, intra-arterial substance P was administered at 4, 8, 16 pmol/min, acetylcholine at 5, 10, 20 μg/min and sodium nitroprusside at 2, 4, 8 μg/min for 10 min at each dose [Newby *et al* 1997a, 1998a]. The drugs were separated by 20 min of saline infusion and administered in a randomised order. All infusions were given at a constant infusion rate of 1 mL/min. Bilateral venous sampling was performed at baseline and during infusion of each substance P dose for fibrinolytic factors assays (as described in Chapter 2.4.2.1).

6.3.4 Data Analysis and Statistics

Estimated net t-PA antigen and activity release has been defined previously in Chapter 2.5.2. Data were examined, where appropriate, by analysis of variance (ANOVA) with repeated measures and two-tailed paired Student's *t*-test. All results are expressed as mean±SEM. Statistical significance was assigned at the 5% level.

6.4 Results

6.4.1 Baseline Characteristics

The baseline characteristics of all subjects are shown in Table 6.1. The patient groups are matched for age, smoking history, diabetes mellitus, hypertension and severity of coronary artery disease although there appeared to be a greater proportion of female subjects in the acute stent thrombosis group. No significant abnormalities were demonstrated in the thrombophilia screen in all study subjects.

6.4.2 Endothelium-dependent Vasomotion

There were no significant changes in heart rate, blood pressure and non-infused forearm blood flow during drug infusion in all studies. Forearm blood flow increased in a dose-dependent manner during substance P, acetylcholine, and sodium nitroprusside infusions ($P<0.05$, ANOVA) (Table 6.2). However, there were no significant differences between the four groups.

6.4.3 Endogenous Fibrinolysis

Compared to the non-infused arm, substance P caused dose-dependent increases in plasma t-PA antigen and activity concentrations in the infused arm in all subjects ($P<0.05$, Table 6.3), but this increase was not different between the groups. Patients with acute stent thrombosis had an apparently higher t-PA antigen release but this did not achieve statistical significance nor was this seen with t-PA activity. Post-hoc analysis demonstrated a significant reduction of t-PA antigen and activity release in current smokers compared to non-smokers ($P<0.05$, Figure 6.1).

Table 6.1 Baseline Characteristics

	Acute Stent Thrombosis (n=6)	**In-stent Restenosis** (n=10)	**Control Patients** (n=10)	**Healthy Subjects** (n=10)
Sex (male:female)	2 : 4	9 : 1	8 : 2	9 : 1
Age	65 ± 3	62 ± 2	63 ± 2	58 ± 3
Current Smoker, % (n)	17 (1)	20 (2)	20 (2)	20 (2)
Diabetes mellitus, % (n)	17 (1)	30 (3)	20 (2)	0 (0)
Hypertension, % (n)	50 (3)	60 (6)	30 (3)	0 (0)
Three-vessel disease, % (n)	33 (2)	30 (3)	20 (2)	0 (0)
Normal or mild left ventricular impairment, % (n)	84 (5)	90 (9)	80 (8)	100 (10)
Average Stent Diameter, mm	3.3±0.2	3.0±0.1	3.2±0.2	
Average Stent Length, mm	16±2	18±2	20±2	
<u>Medical Therapy</u>				
Aspirin, % (n)	100 (6)	100 (10)	100 (10)	0 (0)
β-adrenoceptor antagonists, % (n)	50 (3)	50 (5)	60 (6)	0 (0)
Angiotensin converting enzyme inhibitors, % (n)	33 (2)	60 (6)	40 (4)	0 (0)
Statins, % (n)	100 (6)	80 (8)	80 (8)	0 (0)
Calcium channel blockers, % (n)	50 (3)	60 (6)	10 (1)	0 (0)
Oral Nitrates, % (n)	50 (3)	30 (3)	20 (2)	0 (0)

Mean ± SEM

Table 6.2
Infused forearm blood flow during substance P, acetylcholine and sodium nitroprusside infusion

Drug infusion	Infused Forearm Blood Flow (mL/100 mL/min)			
	Acute Stent Thrombosis (n=6)	**In-stent Restenosis** (n=10)	**Control Patients** (n=10)	**Healthy Subjects** (n=10)
Baseline	1.7 ± 0.4	2.5 ± 0.3	2.4 ± 0.2	2.8 ± 0.5
Substance P (pmol/min)				
4	8.6 ± 0.5	9.6 ± 1.1	9.7 ± 1.2	7.9 ± 1.0
8	9.7 ± 0.7	10.6 ± 1.1	11.0 ± 1.1	9.7 ± 1.3
16	11.6 ± 1.3*	11.3 ± 1.4*	13.2 ± 1.6*	12.4 ± 1.9*
Acetylcholine (μg/min)				
5	7.7 ± 1.8	5.7 ± 1.2	6.2 ± 0.9	6.2 ± 1.3
10	9.7 ± 2.3	6.3 ± 1.4	6.9 ± 1.0	7.3 ± 1.1
20	14.4 ± 3.1‡	9.2 ± 2.0‡	9.0 ± 1.5†	9.4 ± 2.1‡
Sodium nitroprusside (μg/min)				
2	9.7 ± 2.9	7.3 ± 1.0	7.3 ± 0.6	8.4 ± 0.9
4	11.6 ± 2.7	11.1 ± 1.2	10.1 ± 0.9	10.9 ± 1.3
8	13.3 ± 2.6‡	13.4 ± 1.4*	12.6 ± 1.4*	14.4 ± 2.1*

ANOVA for dose response *P<0.0001, †P<0.001, ‡P<0.05

Table 6.3 Plasma tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) concentrations and release during substance P infusion. Mean ± SEM

		Acute Stent Thrombosis (n=6)		In-stent Restenosis (n=10)		Control Patients (n=10)		Healthy Subjects (n=10)	
	Substance P (pmol/min)	**Infused arm**	**Non-infused arm**	**Infused arm**	**Non-infused arm**	**Infused arm**	**Non-infused arm**	**Infused arm**	**Non-infused arm**
t-PA antigen (ng/ml)	**Baseline**	4.9 ± 0.9	4.8 ± 0.9	4.3 ± 0.7	4.1 ± 0.6	4.7 ± 0.3	4.9 ± 0.5	5.1 ± 0.4	5.4 ± 0.5
	4	7.2 ± 1.1	5.2 ± 1.2	5.4 ± 0.8	4.2 ± 0.6	5.8 ± 0.5	4.7 ± 0.4	6.1 ± 0.6	5.4 ± 0.5
	8	7.9 ± 1.1	4.9 ± 1.2	6.0 ± 0.7	4.7 ± 0.6	6.5± 0.6	4.7 ± 0.4	7.0 ± 0.8	5.5 ± 0.5
	16	10.3 ± 0.8*	5.6 ± 1.3	8.8 ± 1.5*	4.7 ± 0.6	7.6 ± 0.8†	4.9 ± 0.4	8.5 ± 1.3	7.6 ± 1.5
Estimated t-PA antigen release (ng/100 mL/min)	**Baseline**	0.1 ± 0.5		0.2 ± 0.4		-0.4 ± 0.4		-0.5 ± 0.4	
	4	11.2 ± 4.1		6.8 ± 2.5		6.9 ± 2.8		3.1 ± 1.3	
	8	18.7 ± 7.7		6.9 ± 2.5		12.2 ± 3.3		7.8 ± 3.3	
	16	34.4 ± 10.1*		25.3 ± 7.5‡		19.6 ± 3.3‡		5.9 ± 2.1*	
Net t-PA antigen release (AUC)		47.1 ± 13.9		26.4 ± 6.6		28.6 ± 8.0		13.6 ± 4.1	
	Baseline	2.1 ± 0.5	1.8 ± 0.4	2.0 ± 0.3	2.1 ± 0.5	1.2 ± 0.2	1.2 ± 0.2	0.8 ± 0.2	1.0 ± 0.2
t-PA activity	**4**	5.3 ± 1.0	2.2 ± 0.4	4.6 ± 0.8	2.5 ± 0.6	2.4 ± 0.5	1.29 ± 0.2	2.3 ± 0.5	1.0 ± 0.2
(IU/mL)	**8**	6.7 ± 1.1	2.1 ± 0.4	4.7 ± 0.9	2.7 ± 0.6	3.0 ± 0.6	1.88 ± 0.3	3.8 ± 1.2	1.4 ± 0.3
	16	7.6 ± 1.1†	3.8 ± 0.8	7.0 ± 0.9‡	3.6 ± 0.9	5.5 ± 1.0‡	2.04 ± 0.4	5.9 ± 1.7	1.7 ± 0.4
Estimated t-PA activity release (IU/100 mL/min)	**Baseline**	0.1 ± 0.2		-0.1 ± 0.4		0.0 ± 0.1		-0.3 ± 0.1	
	4	16.8 ± 4.6		11.1 ± 3.6		6.6 ± 2.8		5.6 ± 1.8	
	8	27.9 ± 5.9		11.3 ± 3.8		6.1 ± 2.3		16.1 ± 8.5	
	16	25.5 ± 5.5†		18.3 ± 4.2†		25.8 ± 5.3‡		31.5 ± 13.6*	
Net tPA activity release (AUC)		57.5 ± 10.9		31.5 ± 8.2		25.6 ± 7.7		37.0 ± 16.7	
PAI-1 antigen (ng/mL)	**Baseline**	33 ± 4	36 ± 4	28 ± 7	30 ± 7	43 ± 8	43 ± 7	36 ± 5	36 ± 5
	16	28 ± 2	33 ± 4	32 ± 7	33 ± 7	42 ± 7	46 ± 8	36 ± 5	34 ± 5

ANOVA for dose response *$P<0.05$, †$P<0.01$, ‡$P<0.001$

AUC - Area under the curve

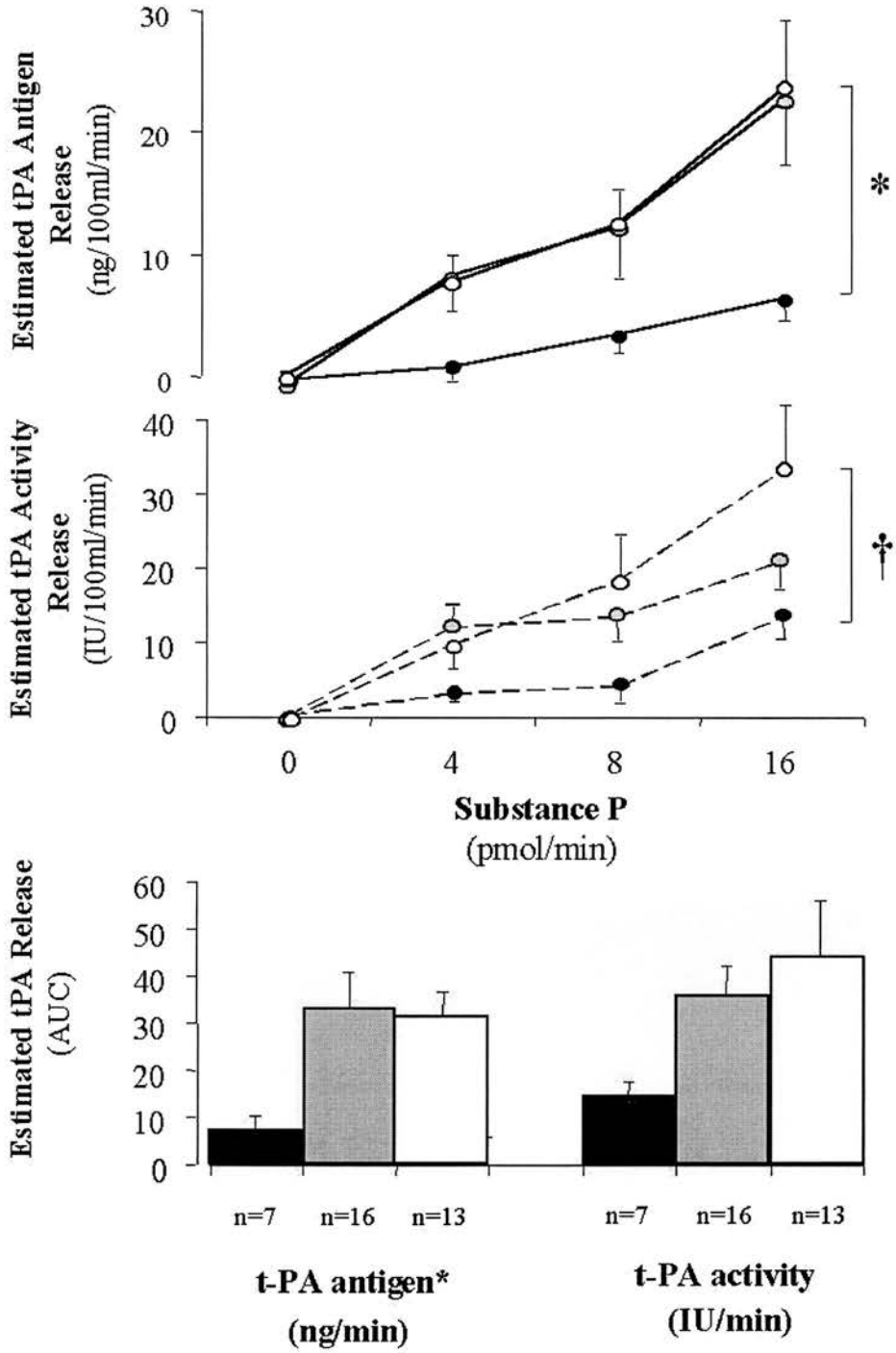

Figure 6.1 Estimated net release of tissue plasminogen activator (t-PA) antigen and activity in current smokers (black), ex-smokers (grey) and non-smokers (white). Lower panel represents area under the curve (AUC) for the response. ANOVA for smoking status *P<0.01; †P<0.05.

6.4.4 Platelet Aggregation

All patients were on aspirin therapy and had reduced platelet aggregation in response to collagen ($P<0.01$) compared to healthy volunteers (Figure 6.2). There was no significant difference in platelet aggregation to thrombin, collagen or U46619 between the patient groups (Figure 6.2). However, platelet aggregation was increased in patients with acute stent thrombosis in response to ADP compared to in-stent restenosis ($P<0.001$), although this was not significant when compared to the control group ($P=0.19$).

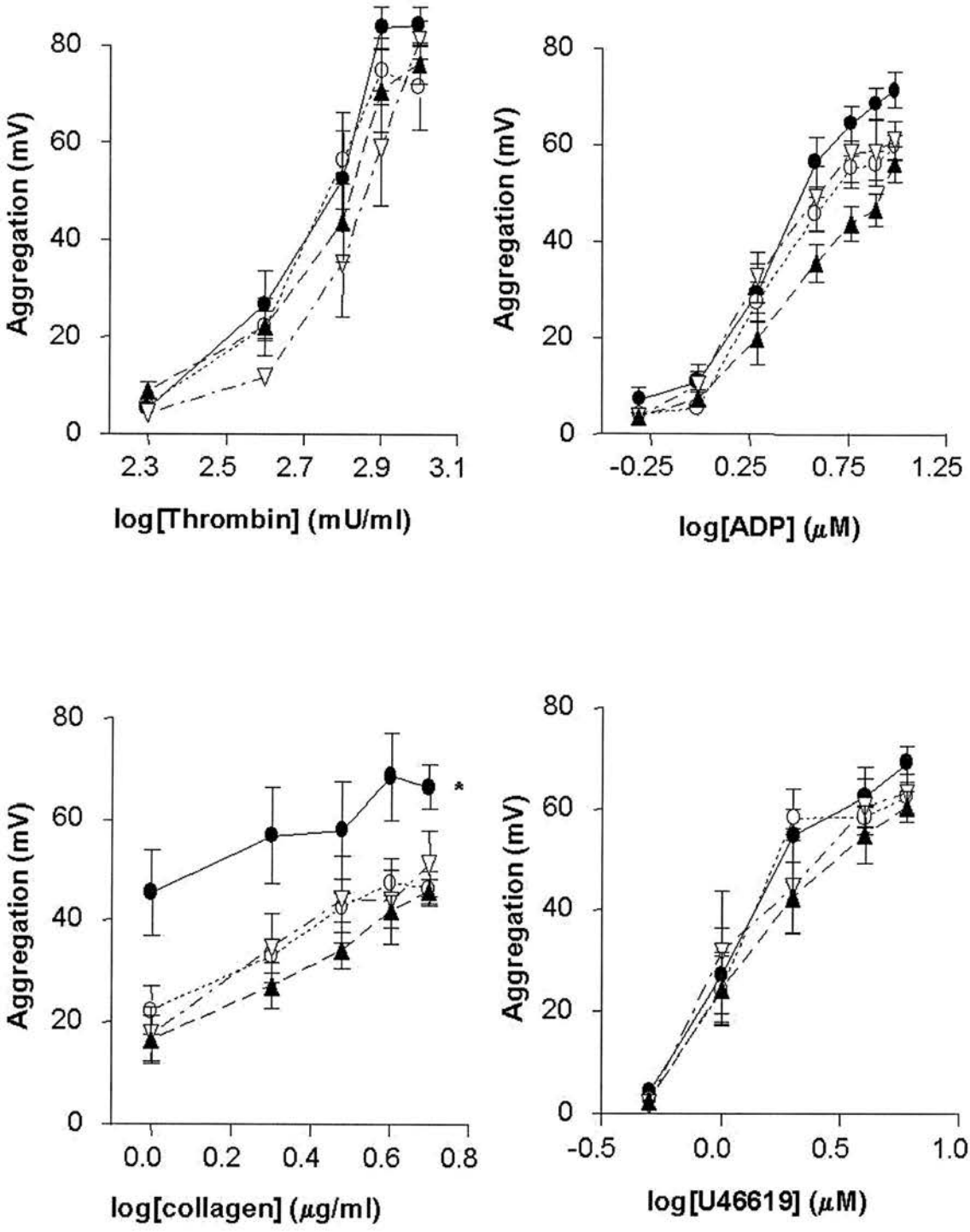

Figure 6.2 Platelet aggregation in response to thrombin, collagen, ADP and U46619 in patients with acute stent thrombosis (open inverted triangles). in-stent restenosis (closed triangles) and uncomplicated PCI with stent implantation (open circles), and healthy matched controls (closed circles). $P<0.001$ for each dose response. *$P<0.01$ Controls vs patient.s.

6.5 Discussion

We have assessed three critical aspects of vascular function in patients who have undergone PCI. We have found no evidence to indicate that endothelial vasomotor or fibrinolytic function plays a major role in the pathogenesis of acute stent thrombosis or in-stent restenosis. Moreover, *in vitro* platelet function appeared to be normal with little evidence of alterations in platelet sensitivity to a number of agonists. In contrast, we have been able to confirm our previous findings [Newby *et al* 1999, 2001] of the marked inhibition of acute t-PA release in current smokers. We conclude that endothelial and platelet functions do not appear to be major determinants of acute stent thrombosis or in-stent restenosis.

6.5.1 Endothelial Vasomotor Function

The endothelium plays a critical role in the regulation of vasomotor tone [Furchgott & Zawadzki 1980; Vanhoutte *et al* 1986] and its injury or dysfunction is an important contributing factor in atherothrombosis. There is progressive impairment of endothelium-dependent vasodilatation with the development of coronary atherosclerosis [Zeiher *et al* 1991] and its associated risk factors, such as hypercholesterolemia [Creager *et al* 1990], smoking [Newby *et al* 1999] and diabetes mellitus [Calver *et al* 1992]. In this study, we assessed forearm blood flow following intra-arterial infusions of the endothelium-dependent vasodilators, substance P and acetylcholine, and the endothelium-independent vasodilator, sodium nitroprusside. We found no differences in the forearm blood flow responses either between the patient groups or comparing the

patient and healthy control groups. Given the absence of risk factors and clinically evident disease, one would have anticipated greater endothelium-dependent vasodilatation in the healthy control subjects. However, ageing has a marked effect on the regulation of basal [Singh *et al* 2002] and stimulated [Egashira *et al* 1993] endothelium-dependent vascular tone and we studied a predominantly elderly population with a mean age of 62 years. Moreover, we recognise that subjects who are apparently healthy in this age group commonly have subclinical atherosclerosis that cannot be excluded in the absence of invasive investigation such as coronary angiography.

6.5.2 Endogenous Fibrinolysis

The regulated release of endothelial t-PA is important mechanism in the defence against intravascular thrombosis especially in the coronary circulation [Rosenberg & Aird 1999]. Several investigators have suggested that impaired endogenous fibrinolysis is associated with restenosis after PCI [Huber *et al* 1992; Kirschstein *et al* 1989], and in particular, a rise in plasma PAI-1 antigen concentrations [Sakata *et al* 1996]. In the present study, we were unable to find any evidence of impaired endogenous fibrinolysis in our patients with no apparent differences in plasma PAI-1 antigen concentrations or acute endothelial t-PA release. However, when stratifying the subjects according to smoking habit, post-hoc analysis indicated that acute t-PA release was markedly impaired in smokers compared to non-smokers. This confirms our previous findings of impaired t-PA release in smokers [Newby *et al* 1999, 2001] and gives support to our conclusions that, rather than a lack of power, there appears to be no major impairment of endogenous fibrinolysis in patients with acute stent thrombosis or in-stent restenosis.

6.5.3 Platelet Function

Platelet adherence to the arterial wall occurs within minutes of arterial injury. Pathological studies [Serruys *et al* 1991] in both porcine coronary arteries and in human saphenous vein grafts have shown that the earliest vascular response to stent implantation is extensive platelet deposition. However, despite important reductions in periprocedural events [The EPILOG Investigators 1997; The EPISTENT Investigators 1998], the glycoprotein IIb/IIIa receptor antagonist abciximab does not reduce in-stent restenosis [The ERASER Investigators 1999]. Consistent with this observation, platelet aggregation in response to thrombin, ADP, the thromboxane A2 analogue U46619 or collagen was unaffected in our study patient populations. In keeping with long-term aspirin use, patients in all three groups demonstrated reduced collagen-dependent platelet aggregation in comparison with healthy subjects. Again, this suggests that, rather than a lack of power, there appears to be no major difference in platelet aggregation in patients with acute stent thrombosis or in-stent restenosis. Although assessment of platelet function should ideally be performed after aspirin has been withheld for at least one week, aspirin use was unavoidable in our patient population.

6.5.4 Study Limitations

There are several potential limitations to our clinical study. First, complications from stent implantation may arise due to procedural difficulties or suboptimal stent deployment. However, we were careful to exclude such patients with overt technical problems during the PCI procedure. Second, acute stent thrombosis is a fortunately rare complication of PCI but this makes recruitment of such patients problematic. This is

reflected in the modest number of patients with acute stent thrombosis in our study and means that we lack sufficient power to address the influence of all the individual variables associated with thrombosis, particularly given the higher proportion of female patients in this group. This relatively low number of patients and subjects in the whole study may also raise the potential possibility of a type II error. Third, the assessment in our study is performed at least six months following PCI and stent implantation in order to exclude the development of late restenosis in the control patient group. Whilst this avoids the influence of acute confounding factors, we may have missed a transient impairment in endothelial or platelet function. It is therefore possible that changes in platelet behaviour, fibrinolysis or vascular function may occur in the acute phase of stent-thrombosis and this study does not definitely exclude that changes in these factors contribute to the development of acute stent thrombosis. Finally, we used a clinical diagnosis of restenosis and cannot exclude a degree of subclinical angiographic restenosis in the control patient group.

6.5.5 Conclusion

In conclusion, our study suggests that endothelial dysfunction, platelet aggregation and endogenous fibrinolysis do not appear to play a major role in the pathogenesis of acute stent thrombosis or in-stent restenosis.

CHAPTER 7

Conclusions and Future Directions

7.1 Vasomotor Effects of Inflammation

In a series of studies, we have explored the mechanistic links between inflammation, endothelial dysfunction and endogenous fibrinolysis. We adopted a previously reported model of mild systemic inflammation [Hingorani *et al* 2000], and also further developed a model of local vascular inflammation *in vivo* in man using unilateral intra-brachial infusion of human recombinant tumour necrosis factor-α (TNF-α). This direct local vascular and endothelial inflammatory response was confirmed by the local rise in plasma interleukin-6 (IL-6) and tissue-type plasminogen activator (t-PA) concentrations. We assessed the effects of systemic and local inflammatory stimuli on endothelial vasomotor and fibrinolytic function and demonstrated their complex effects on endothelial function.

7.1.1 Systemic Inflammation

In a randomised controlled study, we assessed the effects of mild systemic inflammation generated by *Salmonella typhi* vaccination on basal and stimulated forearm blood flow following intra-arterial infusions of endothelium-dependent and -independent vasodilators. Contrary to a previous report [Hingorani *et al* 2000], we demonstrated that mild systemic inflammation was not associated with a major alteration in basal or stimulated vasomotor tone in the forearm circulation.

7.1.2 Inflammatory Cytokines

Although isolated intra-arterial TNF-α, IL-6 or lipopolysaccharide (LPS) infusions had no direct effect on basal forearm blood flow, we demonstrated that TNF-α impaired

resistance vessel endothelium-dependent vasodilatation (to bradykinin and acetylcholine), possibly through the development of acute arterial endothelial injury. The effects of TNF-α were specific for the endothelium since endothelium-independent vasodilatation to the nitric oxide donor, sodium nitroprusside, was unaltered.

TNF-α may alter endothelial vasomotor responses through various mechanisms including decreased constitutive nitric oxide synthase expression, increased inducible nitric oxide synthase (iNOS) expression, and enhanced production of reactive oxygen species [Yoshizumi *et al* 1993; Matsubara *et al* 1986], although the precise mechanism was not elucidated in our studies.

7.2 Endogenous Fibrinolytic Effects of Inflammation

7.2.1 Systemic Inflammation

Acute systemic inflammation generated by vaccination is associated with a two- to threefold augmentation of endothelial bradykinin-induced t-PA release in the absence of systemic haemodynamic effects. The inflammatory stimulus appears to potentiate specifically the storage or acute release of t-PA rather than a generalised upregulation of protein synthesis and basal secretion, as basal plasma concentrations of both t-PA and plasminogen activator inhibitor type 1 (PAI-1) were not affected. This suggests that mild acute inflammation may induce anti-thrombotic properties in the forearm circulation, and may represent an adaptive response to inhibit intravascular thrombus deposition under circumstances of acute vascular inflammation.

7.2.2 Inflammatory Cytokines

Local intra-arterial TNF-α administration is associated with a unique profile of endogenous t-PA release. In contrast to IL-6 and LPS, TNF-α causes a slow-onset, sustained and selective release of t-PA from the vascular endothelium in the absence of demonstrable effect on basal vasomotor tone or plasma von Willebrand factor or PAI-1 antigen concentrations. Moreover, pre-treatment with TNF-α augments acute bradykinin-induced endothelial t-PA release despite reduced endothelium-dependent vasodilatation.

7.2.3 Mechanisms of t-PA Release

Several authors [Newby *et al* 1997a; Brown *et al* 1999; Jern *et al* 1994] have previously reported acute rapid t-PA release during intra-arterial substance P, bradykinin and methacholine infusions, with a near instantaneous onset and offset of t-PA release but with no sustained release after cessation of administration. In contrast, we have demonstrated that TNF-α administration was associated with a unique profile of endothelial t-PA release that was gradual and sustained.

Endothelial cells synthesise and secrete t-PA both constitutively and facultatively, and the acute rapid facultative release of t-PA arises from the translocation of a dynamic intracellular storage pool of t-PA [van den Eijnden-Schrauwen *et al* 1995; Tranquille & Emeis 1989]. Agonists, such as substance P and bradykinin, are likely to stimulate t-PA release via exocytosis of these granules because of the near instantaneous release of t-PA. The profile of t-PA release seen with TNF-α is distinct from this pathway.

Although the exact mechanism was not elucidated in these studies, it is likely to arise from an increase in *de novo* t-PA synthesis and its constitutive release rather than via facultative pathways. However, the initial detectable rise in t-PA release may be too early for protein synthesis to occur, and may be due to other potential mechanisms that include the activation of adherent leukocytes to cause the generation of secondary mediators that enhance t-PA release.

7.3 Vascular Inflammation and Fibrinolysis in Patients with Coronary Artery Disease

Our studies have characterised the complex effects of local vascular inflammation in healthy individuals. Patients with atherosclerosis [Ludmer *et al* 1986] as well as vascular inflammation [Hingorani *et al* 2000; Kharbanda *et al* 2002] exhibit extensive evidence of altered endothelial function, and previous studies have indicated a direct association between elevated plasma fibrinolytic factors and markers of systemic inflammation [Haverkate *et al* 1995]. This raises the question as to whether vascular inflammation and underlying endothelial injury are causally related to the elevation in plasma concentrations of endothelium-derived fibrinolytic factors in coronary artery disease. Moreover, whilst increases in fibrinolytic factors may be expected to protect against the propagation of intravascular thrombosis, it may also reflect widespread endothelial dysfunction. A dominant pro-inflammatory vascular response that occurs with acute plaque rupture may overwhelm any locally protective fibrinolytic effect. Hence the clinical outcome of acute vascular inflammation may depend on the relative

balance between the protective anti-thrombotic actions and potential plaque destabilisation associated with increased vascular t-PA release.

We have confirmed the direct association between plasma t-PA, TNF-α and systemic inflammation in healthy men and these studies will now be extended to investigate this potential causal relationship in patients with stable coronary artery disease using this acute local vascular inflammatory model. Understanding this link can in part explain the adverse prognosis associated with increased plasma t-PA concentrations. Preliminary data have replicated the findings that acute cytokine-induced arterial inflammation impairs vasomotor function whilst enhancing endothelial t-PA release in patients with coronary artery disease [Robinson *et al* 2005].

The association between local vascular inflammation and endogenous fibrinolysis in patients with stable coronary artery disease is the subject of an on-going British Heart Foundation Research Project (FS/2001/047, PG/2001/068).

7.4 Anti-cytokine Therapy in Heart Disease

Data from our work and other authors have illustrated the pivotal role inflammation plays in the pathophysiology of endothelial dysfunction and atherothrombosis. Several studies have also indicated that both the acute coronary syndromes and congestive heart failure are acute and chronic inflammatory states respectively [Ross 1999], with elevated plasma inflammatory mediator concentrations that correlate with disease

severity [Biasucci *et al* 1996, 1999; McMurray 1991]. Therefore, inhibiting the actions of these inflammatory mediators has been proposed to represent potential therapeutic strategies.

7.4.1 Inhibition of Inflammatory Cytokine: TNF-α Antagonism

Tumour necrosis factor-α is implicated in the progression of ischaemic heart disease, and we have shown in Chapter 4 that TNF-α directly impairs endothelial vasomotor function, a finding common to both acute coronary syndromes and heart failure. Tumour necrosis factor-α antagonism is possible with specific cytokine inhibitors. Etanercept is a recombinant human TNF-α receptor that binds to soluble TNF-α and functionally inactivates TNF-α by preventing it from binding to its receptors on cell surface membranes [Deswal *et al* 1999; Mann *et al* 2004]. Infliximab is a recombinant immunoglobulin G1-κ, human-murine chimeric monoclonal antibody that specifically and potently binds to and neutralises soluble TNF-α and its membrane-bound precursor [Chung *et al* 2003]. These drugs have been shown to be effective therapies for psoriasis, ankylosing spondylitis, Crohn's disease and active rheumatoid arthritis when conventional treatments are inadequate.

7.4.2 Anti-cytokine Therapy in Patients with Chronic Heart Failure

In experimental animals, exogenous administration and transgenic overexpression of proinflammatory cytokines could mimic many aspects of the heart failure phenotype, including left ventricular remodelling and reduced survival [Bozkurt *et al* 1998; Kubota *et al* 1997; Sivasubramanian *et al* 2001], leading to the hypothesis that TNF-α

antagonism may be beneficial in heart failure. Preclinical studies indeed demonstrated that TNF-α antagonism reversed some of the deleterious effects of TNF-α *in vitro* and *in vivo*, and preliminary clinical trials also showed an improvement in functional status [Deswal *et al* 1999], increase in left ventricular ejection fraction and decrease in left ventricular volumes [Bozkurt *et al* 2001] in patients with advanced heart failure.

However, two recently published randomised placebo-controlled trials that evaluated targeted anti-cytokine therapy in heart failure have failed to demonstrate any significant benefit. The RENEWAL (Randomized Etanercept Worldwide Evaluation) [Mann *et al* 2004] and ATTACH (Anti-TNF Therapy Against Congestive Heart Failure) [Chung *et al* 2003] trials assessed the effects of etanercept and infliximab on patients with moderately severe chronic heart failure respectively and both have ruled out any clinically relevant benefit on the rate of death or hospitalisation. On the contrary, high doses (10 mg/kg) of infliximab may adversely affect the clinical condition of patients.

The reasons for the lack of benefit in heart failure are not clear. However, it is possible that the short-term benefits of etanercept observed in earlier studies were offset in the long term by the ability of etanercept to stabilise biologically active TNF-α, thereby acting as TNF-α agonists. The observation that a higher proportion of patients who had undergone longer duration of etanercept therapy had a worse outcome was consistent with this view. A further possibility was that sustained lowering of TNF-α may have led to the loss of the beneficial aspects of cytokine signalling which were necessary for cardiovascular homeostasis in heart failure.

7.4.3 Anti-cytokine Therapy in Patients with Acute Coronary Syndrome

The excessive mortality in acute coronary syndromes is attributed primarily to rupture and thrombotic transformation of the atherosclerotic plaque. Inflammation is recognised to be widespread in the coronary and remote vascular beds and plays a critical role in plaque destabilisation and vulnerability. This is evident in the association of elevated inflammatory marker concentrations in acute coronary syndromes with adverse prognosis and recurrent coronary events [Biasucci *et al* 1996; Ridker *et al* 2000a]. Inhibiting the acute effects of cytokine-mediated inflammation may therefore reduce the extent of endothelial injury. In our studies, we have demonstrated that TNF-α not only modifies endothelial vasomotor function, but also augments fibrinolytic function. This suggests that although TNF-α antagonism may improve vascular function in acute inflammation, it can also potentially attenuate fibrinolytic function and aggravate endothelial injury. This remains speculative, thus assessing the effects of TNF-α antagonism on endothelial dysfunction will be of great interest in elucidating the role of inflammation in acute coronary syndromes. Understanding the effects of cytokine antagonism will help ascertain its potential as an alternative therapeutic avenue in ischaemic heart disease.

The effect of TNF-α antagonism with etanercept in patients with unstable angina is the subject of an on-going British Heart Foundation Research Project (PG/2001068).

7.5 Endothelial Function and Endogenous Fibrinolysis in Ischaemic Heart Disease

Endothelial dysfunction is now believed to be an early step in the pathogenesis and pathophysiology of atherosclerosis. Several studies have assessed endothelium-dependent vasomotion and demonstrated that endothelial function is impaired by a number of risk factors associated with coronary artery disease, such as hypercholesterolaemia [Chowienczyk *et al* 1992], diabetes mellitus [Calver *et al* 1992] and smoking [Newby *et al* 1999]. Recent data have also indicated that in patients with established coronary artery disease, homocysteine is a significant predictor of mortality independent of other traditional risk factors, and is associated with abnormal endothelial vasomotion [Nygard *et al* 1997; Anderson *et al* 2000]. However, endothelial regulation of vascular tone is but one of the many aspects of endothelial function and represents a surrogate marker for the role of the endothelium in atherothrombosis. We have therefore extended these investigations and assessed endothelial fibrinolytic capacity in patients with hyperhomocysteinaemia, and in patients with thrombotic complications following percutaneous coronary intervention (PCI) with stent implantation.

7.5.1 Patients with Recent Myocardial Infarction and Hyperhomocysteinaemia

In patients with recent myocardial infarction, we demonstrated that elevation of plasma homocysteine concentration is associated with impaired endothelium-dependent vasodilatation. However, endogenous fibrinolysis is not affected in this group of patients. Furthermore, we have shown that this endothelial vasomotor dysfunction is not rectified by vitamin supplementation with folate, pyridoxine and cyanocobalamin.

These results provide further evidence for the role of homocysteine in vascular damage, but do not support the hypothesis that vitamin supplementation improves endothelial function in patients with established coronary artery disease.

Although earlier studies have reported a potential beneficial effect on cardiovascular outcome following coronary angioplasty [Schnyder *et al* 2002], our findings are consistent with recent data that showed vitamin supplementation failed to reduce adverse events. On the contrary, folate therapy may paradoxically lead to an increase in restenosis following coronary stent implantation [Lange *et al* 2004]. Thus the role of vitamin therapy in cardiovascular disease remains controversial, and more prospective data are needed before any recommendations can be made at all regarding the use of vitamin supplementation in coronary artery disease. Several multi-centre randomised controlled trials are under way and will provide useful answers to these issues within the next few years [Mangoni & Jackson 2002].

7.5.2 Patients with Acute Stent Thrombosis or In-stent Restenosis

Acute stent thrombosis and in-stent restenosis are serious complications of percutaneous coronary intervention and may be associated with vascular or platelet abnormalities. We assessed three critical aspects of vascular function in patients who have undergone percutaneous coronary intervention. We have found no evidence to indicate that endothelial vasomotor or fibrinolytic function plays a major role in the pathogenesis of acute stent thrombosis or in-stent restenosis. Moreover, *in vitro* platelet function appeared to be normal with little evidence of alterations in platelet sensitivity to a

number of agonists. We conclude that endothelial and platelet functions do not appear to be major determinants of acute stent thrombosis or in-stent restenosis.

7.6 Future Directions

In addition to our work on the direct role of inflammation on endothelial function in patients with heart disease, there are other regulatory mechanisms that contribute to the control of vascular tone and fibrinolytic function. Furthermore, the release of inflammatory mediators can lead to the activation of several vasoactive compounds that can lead to vascular dysfunction evident in a number of inflammatory conditions including sepsis and endotoxaemia. Regulatory mechanisms that may influence this dynamic relationship between inflammation and endothelial function, as well as other potential therapeutic candidates warrant further investigation.

7.6.1 Role of Glucocorticoid Metabolism in Local Vascular Inflammation

Both inflammation and endothelial function are regulated by glucocorticoids. Elevated circulating levels of glucocorticoids, for example in Cushing's syndrome, cause many of the risk factors for cardiovascular disease, including hypertension, hyperglycaemia, dyslipidaemia and central obesity. A long-term influence of glucocorticoids on endogenous basal plasma fibrinolytic variables and von Willebrand factor has also been described [Fatti *et al* 2000]. The effects of glucocorticoid on vascular tone are well established and include potentiation of vasoconstrictor responses to noradrenaline and impaired endothelium-dependent vasodilatation [Ullian 1999].

Glucocorticoids are classically anti-inflammatory and their role during physical stress is to modify the acute inflammatory response to injury or infection, which they achieve by diverse effects on humoral and cellular immunity. However, attempts to exploit these actions in the blood vessel wall by administering short-term methylprednisolone therapy in patients with unstable angina or following coronary artery stent insertion have failed to show any beneficial effects [Azar *et al* 2000; Reimers *et al* 1998]. Conversely, such therapy has been associated with a potentially adverse outcome. Whether these disappointing clinical results reflect systemic or local intra-vascular effects of glucocorticoids remain unclear. Within vessel walls, it is likely that there is a balance between beneficial as well as adverse effects of glucocorticoid, which can be modulated independent of their systemic effects. Thus local control of vascular responses to glucocorticoid may potentially be more important than variations in systemic cortisol concentrations in influencing the progression of vascular lesions.

Glucocorticoid availability is regulated locally within the vascular wall by two key enzymes, 11β-hydroxysteroid dehydrogenase types 1 and 2. Regulation of these enzymes by TNF-α and other cytokines predicts that vascular inflammation is associated with increased local glucocorticoid levels [Thieringer *et al* 2001; Heiniger *et al* 2001], which may be important not only to counter-regulate the inflammatory response, but also to mediate changes in endothelial function. Both these enzymes are altered in subjects with cardiovascular risk factors, and animal models suggest that these changes are reflected in the vessel wall [Takeda *et al* 1998]. Thus pathological

variation in glucocorticoid availability may contribute to variations in response to inflammation and hence to the development and progression of disease.

Understanding the mediation and counter-regulation of vascular inflammation by glucocorticoids may allow new approaches to modifying the development and progression of cardiovascular disease. Using murine and clinical models, the determination of the effects of local glucocorticoid metabolism in the vascular wall on local endothelial vasomotor and fibrinolytic function during vascular inflammation is the subject of an ongoing British Heart Foundation Research Project (PG/02/113/14452).

7.6.2 Anti-inflammatory Strategies

7.6.2.1 Lessons From Anti-cytokine Therapy in Heart Failure

In spite of promising preclinical and preliminary data suggesting that anti-TNF-α strategies may be beneficial to cardiac performance, two recent large multi-centre randomised controlled clinical studies in patients with symptomatic heart failure (ATTACH, RENEWAL) have failed to demonstrate salutary benefits [Chung *et al* 2003; Mann *et al* 2004]. Although the reasons for these disappointing results were not clear, one possible explanation was that while excessive levels of inflammatory cytokines such as TNF-α may be harmful, reduced levels of these mediators may also have adverse effects on the myocardium reflecting the involvement of these cytokines in both maladaptive and adaptive responses [Meldrum 1998]. Our studies lend support to this hypothesis, as we have shown that although acute vascular inflammation with TNF-

α directly impairs endothelium-dependent vasodilatation, it also augments the acute fibrinolytic capacity. This suggests that vascular inflammation may also have a protective effect on the endothelium. Therefore, whilst anti-cytokine therapy has been shown to improve vasomotor function, fibrinolytic function may potentially be impaired, and could account in part for the lack of benefit in ATTACH and RENEWAL. It is however not known whether targeting acute inflammation in acute coronary syndromes would alter the balance of vasomotor and fibrinolytic function. Therefore, exploring the interaction between endothelial function and acute vascular inflammation in cardiovascular disease could help answer this conundrum, an issue which would be addressed in an on-going British Heart Foundation Research Project (PG/2001068).

7.6.2.2 Future Possibilities

Although results from the initial trials with etanercept and infliximab in heart failure were disappointing, a better understanding of the complexities that might impinge on the use of these agents may still hold some promise for the potential application of anti-cytokine strategies in left ventricular dysfunction [Aukrust *et al* 2004; Feldman & McTiernan 2004]. Animal studies suggest that patient selection and possibly gender may be important factors in identifying patients who are likely to benefit. It is also probable that patients with earlier disease are more likely to achieve a beneficial long-term response. Anti-TNF-α therapy might also be beneficial in patients with acute decompensation in whom elevated cytokine levels produce a negative inotropic effect. Thus these observations may serve as a platform for future evaluation of the efficacy of anti-TNF-α therapies.

7.6.2.3 Other Potential Immunomodulators

Apart from cytokine antagonists, other potential immunomodulators have been assessed in congestive heart failure [Sliwa *et al* 1998]. Pentoxifylline, a xanthine-derived agent known to inhibit the production of TNF-α, was the first immunomodulatory agent that showed beneficial effects in heart failure patients. Although there was improvement in symptoms and left ventricular dysfunction, this was not accompanied by a decrease in plasma levels of Fas, a member of the TNF receptor super family. Thus the potential benefits of pentoxifylline treatment may not be directly related to its anti-cytokine properties. Thalidomide has both anti-inflammatory and anti-oncogenic properties, and interestingly, was recently shown to improve left ventricular ejection fraction in congestive heart failure, accompanied by a marked decrease in plasma levels of TNF-α [Gullestad *et al* 2002]. However, its use will probably be limited by its anti-angiogenic properties. Therapy with intravenous immunoglobulin has recently been shown to enhance left ventricular ejection fraction in patients with congestive heart failure independent of the aetiology [Gullestad *et al* 2001]. Intravenous immunoglobulin influences the levels of several cytokines and cytokine modulators, resulting in down-regulation of inflammatory responses. Clinical application of this therapy will require further evaluation in large randomised trials.

Other potential candidates that have been investigated in animal models include interleukin-10, interleukin-1 receptor antagonist as well as activators of peroxisome proliferators activated receptors (PPPARs). Further research in this area will be

required to identify the role of these important factors in the immunopathogenesis of acute coronary syndromes and congestive heart failure.

7.6.3 Role of Inducible Nitric Oxide Synthase (iNOS) in Vasoregulation

Nitric oxide is a key mediator of vascular tone, and excessive synthesis of nitric oxide by inducible nitric oxide synthase (iNOS), activated in response to inflammatory mediators, may account for the vascular dysfunction evident in a number of inflammatory conditions including sepsis and endotoxaemia. Congestive heart failure is a chronic inflammatory disease with evidence of endothelium-dependent vasomotor dysfunction and possible altered basal nitric oxide release. It is however not known whether induction of iNOS in heart failure provides the link between inflammation and the alteration in vascular tone.

Until recently, it has not been possible to distinguish clearly between constitutive and inducible NOS activity due to the lack of specificity of inhibitors. N-(3-(Aminomethyl) benzyl) acetamidine (1400W) is a novel selective inhibitor of human iNOS that has recently become available for clinical use [Garvey *et al* 1997; Boer *et al* 2000]. It competes with L-arginine to bind irreversibly with iNOS and is at least 5000-fold more selective for iNOS than eNOS making it the most selective iNOS inhibitor to date.

Therefore, assessing the contribution of functional iNOS activity to peripheral vascular tone in patients with symptomatic heart failure will help us understand the role of nitric oxide in vascular inflammation and endothelial dysfunction, and also identify potential therapeutic targets.

The role of iNOS activity in the maintenance of peripheral vascular tone in severe chronic heart failure is the subject of an on-going British Heart Foundation Research Project (FS/2001049).

7.7 Clinical Relevance

A consistent link between endothelial dysfunction, inflammation and cardiovascular disease is now well established, although most studies have focused predominantly on endothelium-dependent vasomotor function. We have demonstrated that the interaction between inflammation and endothelial function is more complex than previously appreciated. In particular, the effects of TNF-α are diverse which may explain some of the discordant clinical findings where marked improvement in endothelial vasomotion following TNF-α antagonism failed to translate into clinical benefits in patients with heart failure. Our findings therefore suggest that a more selective strategy in anti-cytokine therapy would be necessary, an approach that is currently investigated in an ongoing British Heart Foundation Research Project (PG/2001068).

The importance of endogenous fibrinolysis has been highlighted in recent studies, and reduction in t-PA-activity or release is associated with increased incidence of major adverse cardiac events in patients with acute coronary syndromes. Our studies indicated that there were no significant association between fibrinolysis and hyperhomocysteinaemia, coronary stent complications. However, the unique role of

systemic inflammation and specifically, TNF-α has been identified. Therefore modulating cytokine actions and their interaction with fibrinolysis may be critical in the prevention of thrombotic coronary occlusion and myocardial ischaemia as well as in the future development of anti-thrombotic therapies.

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Abstracts

1. **Chia S**, Reid K, Dawson P, Ludlam CA, Fox KAA and Newby DE. Acute Systemic Inflammation Enhances Tissue Plasminogen Activator Release in Man. *51st Annual Scientific Session, American College of Cardiology, Atlanta, 2002 and 14th World Congress of Cardiology, Sydney, 2002.*

2. **Chia S**, Megson I, Harding SA, Ludlam CA, Fox KAA, Boon N and Newby DE. Endothelial Dysfunction and Impaired Endogenous Fibrinolysis Do Not Contribute To Acute Stent Thrombosis or In-stent Restenosis. *14th World Congress of Cardiology, Sydney, 2002.*

3. **Chia S**, Newton R, Qadan M, Fox KAA, and Newby DE. Intra-arterial Tumour Necrosis Factor-α Impairs Endothelium-Dependent Vasodilatation *In Vivo* In Man. *American Heart Association Scientific Sessions, Chicago, 2002.*

4. **Chia S**, Qadan M, Newton R, Ludlam CA, Fox KA, Newby DE. Tumor Necrosis Factor-α Impairs Endothelium-Dependent Vasodilatation And Stimulates Local Tissue Plasminogen Activator Release in Man: Diverse Vascular Actions of a Pleiotropic Cytokine. *52nd Annual Scientific Session, American College of Cardiology, Chicago, 2003.*

5. **Chia S**, Wilson R, Webb DJ, Fox KA, Ludlam CA, Flapan AD, Newby DE. Impaired Endothelium-dependent Vasomotion In Patients With Recent Myocardial Infarction And Hyperhomocysteinemia. *52nd Annual Scientific Session, American College of Cardiology, Chicago, 2003.*

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7. Dover A, **Chia S**, Cruden NL, Ferguson, Fox KA, Newby DE. Inducible Nitric Oxide Synthase Activity Does Not Contribute to the Maintenance of Peripheral Vascular Tone in Patients With Symptomatic Congestive Heart Failure. *53rd Annual Scientific Session, American College of Cardiology, New Orleans, 2004.*

Appendix

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Acute Systemic Inflammation Enhances Endothelium-Dependent Tissue Plasminogen Activator Release in Men

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OBJECTIVES The purpose of this study was to investigate in vivo the effects of acute systemic inflammation on the endogenous fibrinolytic capacity in men.

BACKGROUND Systemic inflammation and endogenous fibrinolysis play a major role in the pathogenesis of coronary artery disease. Although previous studies have shown impaired endothelium-dependent vasomotor function, the effects of inflammation on the endothelial release of the fibrinolytic factor tissue plasminogen activator (t-PA) are unknown.

METHODS In a double-blind randomized placebo-controlled crossover trial, we administered a mild inflammatory stimulus, *Salmonella typhi* vaccine, or saline placebo to eight healthy men on two separate occasions. Six hours after vaccination, blood flow and plasma fibrinolytic variables were measured in both arms during intrabrachial infusions of bradykinin (40 to 1,000 pmol/min), acetylcholine (5 to 20 μg/min), and sodium nitroprusside (2 to 8 μg/min).

RESULTS Compared with placebo, the *S. typhi* vaccination caused a rise in white cell count (11.1 ± 0.5 $\times 10^9$/l vs. 7.9 ± 0.8 $\times 10^9$/l; $p = 0.004$) and plasma interleukin-6 concentrations (6.9 ± 1.4 pg/ml vs. 1.6 ± 0.4 pg/ml; $p = 0.01$) in addition to a significant augmentation of t-PA antigen (45 ± 9 ng/100 ml/min at peak dose vs. 24 ± 8 ng/100 ml/min at peak dose; $p = 0.016$, analysis of variance) and activity (104 ± 15 IU/100 ml/min vs. 54 ± 12 IU/100 ml/min; $p = 0.006$, analysis of variance) release during bradykinin infusion. Forearm blood flow increased in a dose-dependent manner after bradykinin, acetylcholine and sodium nitroprusside infusions ($p < 0.001$), but this was unaffected by vaccination.

CONCLUSIONS Our results showed that acute systemic inflammation augmented local forearm t-PA release in men, which suggests that acute inflammation may invoke a protective response by enhancing the acute endogenous fibrinolytic capacity in healthy men. Further studies are needed to clarify whether this response is impaired in patients with cardiovascular disease. (J Am Coll Cardiol 2003;41:333–9) © 2003 by the American College of Cardiology Foundation

Atherosclerosis is widely recognized to be an inflammatory disease process involving dysfunction of the vascular endothelium (1). This dysfunction leads to increased expression of leukocyte adhesion molecules, reduced anticoagulant activity and the release of growth factors, inflammatory mediators, and cytokines. Continued inflammation leads to leukocyte and monocyte recruitment, arterial damage, and atherogenesis. Additional cycles of damage cause plaque expansion and disruption that may lead to angina, crescendo angina, and acute coronary syndromes.

Recent epidemiological and observational studies have suggested a link between systemic inflammation and coronary artery disease. Infections by organisms, such as *Chlamydia pneumoniae* and herpes simplex virus type 1, appear to be associated with an increased risk of cardiovascular mortality (2), and approximately 4% of bacteremic patients will develop acute myocardial infarction within a month of infection (3). Increased cardiovascular mortality also is seen after respiratory tract infections (4), severe illnesses requiring intensive care (5), and surgery (6). Markers of systemic inflammation, such as C-reactive protein (CRP), serum amyloid A, interleukin-6 (IL-6), and tumor necrosis factor-alpha, are elevated in patients with cardiovascular disease and are associated with an adverse prognosis and recurrent coronary events (7–10). Moreover, in previously healthy individuals, elevated plasma CRP and IL-6 concentrations predict the development of cardiovascular disease (11–13). Indeed, reflecting its anti-inflammatory action, the preventative benefits of aspirin in reducing cardiovascular risk are proportional to the plasma CRP concentration (11). These data collectively suggest two patterns of association: a link between chronic inflammation and the slow process of atherogenesis and an association between acute systemic inflammation and a transiently increased risk of an acute cardiovascular event.

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Abbreviations and Acronyms

ANOVA = analysis of variance
CRP = C-reactive protein
FBF = forearm blood flow
IL-6 = interleukin-6
PAI-1 = plasminogen activator inhibitor type 1
t-PA = tissue-type plasminogen activator

The vascular endothelium plays a vital role in the control of blood flow, hemostasis, fibrinolysis, and inflammation (14), and impaired endothelial function is implicated in the pathogenesis of coronary artery disease. Tissue plasminogen activator (t-PA) is a fibrinolytic factor released from the endothelium through the translocation of a dynamic intracellular storage pool and regulates the degradation of intravascular fibrin (15). If endogenous fibrinolysis is to be effective, then the rapid mobilization of t-PA from the endothelium is essential because thrombus dissolution is much more effective if t-PA is incorporated during, rather than after, thrombus formation (16,17). However, in the presence of pro-inflammatory states or an imbalance in endogenous fibrinolysis, such microthrombi may propagate, ultimately leading to arterial occlusion and tissue infarction (18).

We have previously described an in vivo model to assess the acute release of t-PA in men (19) and demonstrated an association between t-PA release and endothelial dysfunction (20,21). Hingorani et al. (22) also have recently shown that acute inflammation causes dysfunction of endothelium-dependent vasodilation in humans. However, there have been no studies to assess directly the acute local fibrinolytic capacity after acute inflammation. Therefore, the aim of this study was to test the hypothesis that the acute fibrinolytic capacity is altered by a mild systemic inflammatory response generated by typhoid vaccination.

METHODS

Subjects. Eight healthy nonsmoking men between 20 and 27 years of age 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 and vascular or coronary artery disease. None of the subjects had undergone typhoid vaccination in the previous year or received vasoactive or nonsteroidal anti-inflammatory drugs in the week before the study. All subjects abstained from alcohol for 24 h and from food and caffeine-containing drinks for at least 4 h before each forearm study. All studies were performed in a quiet, temperature-controlled room maintained at 22 to 25°C.

Drugs. An inflammatory response was generated with a typhoid vaccination using *Salmonella typhi* capsular polysaccharide vaccine 0.025 mg (Typhim Vi, Aventis Pasteur MSD, Berkshire, United Kingdom). Pharmaceutical-grade bradykinin (Clinalfa, Läufelfingen, Switzerland), acetylcholine (Clinalfa), and sodium nitroprusside (David Bull Laboratories, Faulding, United Kingdom) were administered after dissolution in saline (0.9%; Baxter Healthcare Ltd., Berkshire, United Kingdom). All solutions were freshly prepared on the day of study.

Hemodynamic measurements. Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges as previously described (19–21). 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., Tokyo, Japan).

Blood sampling and assays. Blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool Stabilyte, Umeå, Sweden) for t-PA assays and trisodium citrate, ethylene diamine tetraacetic acid, and serum bead tubes (Monovette, Sarstedt, Nümbrecht, Germany) for plasminogen activator inhibitor type 1 (PAI-1), IL-6, and CRP assays, respectively, 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 t-PA, PAI-1, IL-6, and CRP concentrations were determined using specific enzyme-linked immunosorbent assays (Coaliza t-PA and Coaliza PAI-1, Chromogenix AB, Mölndal, Sweden; Quantikine human IL-6 immunoassays, R&D Systems, United Kingdom; C-reactive protein enzyme-linked immunosorbent assay, Eurogenetics, Belgium, respectively) and plasma t-PA activity using a photometric method (Coatest t-PA, Chromogenix AB, Mölndal, Sweden). Hematocrit and white cell count were determined using an automated Coulter counter (Beckman-Coulter ACt.8 Coulter Counter, High Wycombe, United Kingdom).

Study design. The *S. typhi* vaccine or saline placebo were injected into the deltoid muscle of each subject's dominant arm at 8:30 AM in a randomized, balanced block, double-blind crossover manner at least two weeks apart. Previous reports have indicated that vaccination-induced endothelial dysfunction is transient and resolves within 32 h (22).

Six hours after vaccination and after a 4-h fast, strain gauges and cuffs were applied. The brachial artery of each subject's nondominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd., Birmingham, United Kingdom) under 1% lidocaine (Xylocaine; Astra Pharmaceuticals, Wayne Pennsylvania) local anesthesia and attached to a 16-gauge epidural catheter (Portex Ltd., Hythe, United Kingdom). Patency was maintained by infusion of saline via a MS2000 syringe infusion pump (Graesby Medical, Watford, United Kingdom). Venous 17-gauge cannulae were inserted into large subcutaneous veins of the antecubital fossae of both arms. Forearm blood flow (FBF) was measured every 6 to 10 min. After 30 min of equilibration with saline infusion, intra-arterial

bradykinin was administered at 40, 200, and 1,000 pmol/min for 10 min at each dose, acetylcholine at 5, 10, and 20 μg/min, and sodium nitroprusside at 2, 4, and 8 μg/min for 6 min at each dose. The drugs were separated by 20 min of saline infusion and administered in a randomized order that was kept constant for each individual. Venous samples were taken at baseline and during infusion of each bradykinin dose but not during sodium nitroprusside or acetylcholine infusion because they do not affect plasma t-PA or PAI-1 concentrations in this forearm model (19,23). White cell count, hematocrit, IL-6, and CRP were determined 6 h after vaccination and hematocrit was repeated at the end of the forearm study.

Data analysis and statistics. Plethysmographic data were extracted from the chart data files, and FBF was calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Microsoft Excel 97). Recordings for the first 60 s after wrist cuff inflation were not used because of the variability in blood flow this initial inflation causes. The last five linear plethysmographic recordings in each 3-min measurement period were calculated and averaged for each arm. The estimated net release of t-PA antigen and activity was defined previously (19) as the product of the infused forearm plasma flow (based on the mean hematocrit and the infused FBF) and the concentration difference between the infused ($[t\text{-}PA]_{inf}$) and noninfused ($[t\text{-}PA]_{noninf}$) arms and is shown as follows:

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

The area under the curve (AUC) was calculated for estimated net t-PA antigen and activity release during bradykinin infusion.

Data were examined, where appropriate, by analysis of variance (ANOVA) with repeated measures and two-tailed paired Student *t* test using Excel 97 (Microsoft). All results are expressed as mean ± SEM. Statistical significance was assigned at the 5% level.

RESULTS

Inflammatory response. All subjects remained healthy throughout the study and reported no localized discomfort or systemic side effects after vaccination. Compared with placebo injection, there was a marked elevation in white cell count ($11.1 \pm 0.5 \times 10^9$/l vs. $7.9 \pm 0.8 \times 10^9$/l; p = 0.004, *t* test) and plasma IL-6 concentrations (6.9 ± 1.4 pg/ml vs. 1.6 ± 0.4 pg/ml; p = 0.01, *t* test) 6 h after typhoid vaccination, although serum CRP concentrations (1.8 ± 1.2 μg/ml vs. 1.0 ± 0.6 μg/ml; p = NS, *t* test) and temperature (p = NS, *t*-test) (Table 1) were unchanged.

Assessment of endothelium-dependent vasomotion. There were no significant effects of vaccination on heart rate, blood pressure, or baseline FBF. There were no significant changes in heart rate, blood pressure, or noninfused FBF during drug infusion on either study day.

Table 1. Baseline Characteristics

	Vaccination	Placebo
Temperature (°C)	37.0 ± 0.1	36.9 ± 0.1
Systolic blood pressure (mm Hg)	131 ± 4	129 ± 5
Diastolic blood pressure (mm Hg)	62 ± 3	66 ± 3
Heart rate (beats/min)	57 ± 1	57 ± 2
Hematocrit	0.41 ± 0.01	0.41 ± 0.01
Infused forearm blood flow (ml/100 ml tissue/min)	3.0 ± 0.6	2.8 ± 0.6

Forearm blood flow increased in a dose-dependent manner during bradykinin, acetylcholine, and sodium nitroprusside infusions (p < 0.001, ANOVA), but there was no change in the blood flow response after vaccination (p = NS, ANOVA) (Fig. 1).

Assessment of fibrinolytic activity. After vaccination, there were no changes in baseline plasma t-PA and PAI-1 antigen concentrations nor plasma t-PA activity concentration. Compared with the noninfused arm, bradykinin caused dose-dependent increases in plasma t-PA antigen and activity (p < 0.001 for both, ANOVA) concentrations in the infused arm that were significantly higher after vaccination (p < 0.03, ANOVA) (Table 2). The *S. typhi* vaccination caused a significant augmentation in the net release of t-PA antigen (45 ± 9 vs. 24 ± 8 ng/100 ml/min at peak dose; p = 0.016, ANOVA) and activity (104 ± 15 vs. 54 ± 12 IU/100 ml/min; p = 0.006, ANOVA) during bradykinin infusion, and a two- to threefold increase in the AUC for net t-PA antigen (37 ± 12 ng/100 ml/min vs. 13 ± 9 ng/100 ml/min; p = 0.14, *t* test) and activity (70 ± 12 IU/100 ml/min vs. 36 ± 8 IU/100 ml/min; p = 0.037, *t* test) release compared with placebo (Fig. 2). Plasma PAI-1

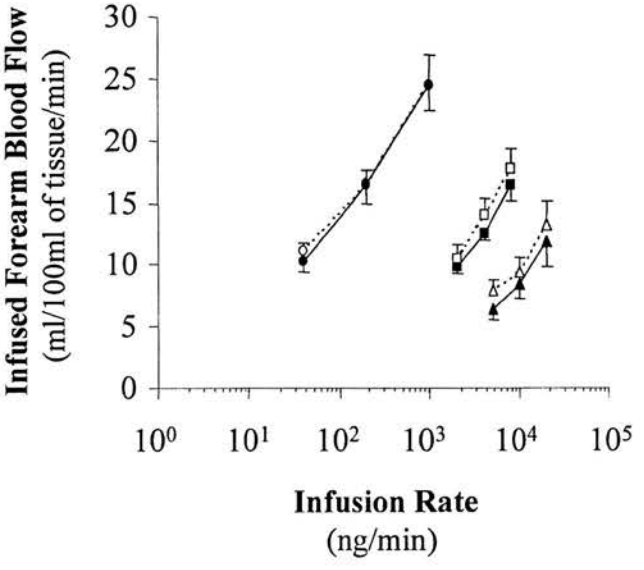

Figure 1. Infused forearm blood flow during bradykinin **(circles)**, acetylcholine **(triangles)**, and sodium nitroprusside **(squares)** infusions in subjects who were administered typhoid vaccination **(closed symbols)** and saline placebo **(open symbols)**. Analysis of variance, p < 0.001 for all (dose-response).

Table 2. Plasma t-PA and PAI-1 Antigen and t-PA Activity/Concentrations

Bradykinin Infusion (pmol/min)	Typhoid Vaccination				Placebo Injection			
	0	40	200	1,000	0	40	200	1,000
t-PA antigen (ng/ml)								
Noninfused arm	2.3 ± 0.3	2.7 ± 0.5	2.9 ± 0.5	3.5 ± 0.5	2.8 ± 0.6	3.2 ± 0.8	3.6 ± 0.7	4.4 ± 0.8
Infused arm	3.0 ± 0.5	3.2 ± 0.5	3.9 ± 0.5	6.7 ± 0.7*	2.8 ± 0.6	3.0 ± 0.7	3.8 ± 0.8	6.1 ± 0.9*
Concentration differences between forearms	0.7 ± 0.4	0.5 ± 0.4	1.0 ± 0.6	3.3 ± 0.8§	0.0 ± 0.2	−0.1 ± 0.4	0.2 ± 0.3	1.7 ± 0.5§‡
t-PA activity (IU/ml)								
Noninfused arm	1.3 ± 0.2	1.4 ± 0.2	1.7 ± 0.2	2.6 ± 0.4	1.1 ± 0.2	1.2 ± 0.2	1.5 ± 0.2	2.5 ± 0.5
Infused arm	1.1 ± 0.2	1.6 ± 0.3	3.3 ± 0.6	9.9 ± 1.2*	1.0 ± 0.2	1.3 ± 0.2	2.5 ± 0.5	6.5 ± 1.3*†
Concentration differences between forearms	−0.2 ± 0.1	0.2 ± 0.1	1.6 ± 0.5	7.4 ± 1.0*	−0.1 ± 0.1	0.1 ± 0.1	0.9 ± 0.5	3.9 ± 1.0*‡
PAI antigen (ng/ml)								
Noninfused arm	17 ± 4			16 ± 2	15 ± 2			16 ± 3
Infused arm	18 ± 5			14 ± 2	16 ± 3			15 ± 2
Concentration differences between forearms	1.3 ± 2.4			−1.2 ± 1.3	0.4 ± 0.8			−0.5 ± 1.7

One-way analysis of variance (ANOVA): *$p < 0.001$, §$p < 0.01$ (dose-response). Two-way ANOVA: †$p = 0.026$, ‡$p < 0.01$ (vaccination vs placebo); $p < 0.001$ for all (dose-response).
PAI-1 = plasminogen activator inhibitor type 1; t-PA = tissue-type plasminogen activator.

concentrations did not change during bradykinin infusion (Table 2).

DISCUSSION

We have demonstrated that an acute mild systemic inflammatory stimulus causes potentiation of bradykinin-induced t-PA release. We conclude that acute systemic inflammation enhances local endothelial t-PA release in men. This may reflect an adaptive mechanism of the vascular endothelium to augment its fibrinolytic response under circumstances of acute inflammation.

Endothelium-dependent vasomotion. We assessed basal and stimulated FBF after intra-arterial infusions of the endothelium-dependent vasodilators bradykinin and acetylcholine, and the endothelium-independent vasodilator sodium nitroprusside 6 to 8 h after vaccination. Bradykinin and acetylcholine have both been widely used to investigate the function of vascular endothelium. Impaired arterial vasodilatory response to endothelium-dependent agonists has been shown in patients with hypertension (24), diabetes mellitus (25), and hypercholesterolemia (26). Hingorani et al. (22) have previously reported that *S. typhi* vaccinations generated an inflammatory response that was associated with a temporary suppression of endothelium-dependent vasodilation in the forearm circulation of six healthy volunteers. Although we applied a similar protocol to their study and included overlapping doses of both bradykinin and acetylcholine, we did not replicate their findings of impaired forearm endothelium-dependent vasodilation after vaccination at 6 to 8 h (Fig. 1). This discrepancy may be partly explained by the variability in vasodilatory response with acetylcholine (27) and the higher maximal vasodilator dose used in our studies, although we used a larger sample size and a double-blind, randomized, placebo-controlled, crossover trial.

Endogenous fibrinolysis. Tissue plasminogen activator, the key enzyme in the initiation of fibrinolysis, is synthesized in endothelial cells and stored in small, dense vesicles. It is secreted both basally and in response to thrombin and several vasoactive agents through a calcium-dependent and G protein-coupled pathway (28). The regulated endogenous release of t-PA plays a major role in the defense against intravascular thrombosis, especially in the coronary circulation (18). Bradykinin is a vasoactive peptide and potent stimulant for the acute release of t-PA from the vasculature (23,29–31) and is produced locally through activation of the kallikrein-kinin system on the surface of endothelial cells (32). In the present study, bradykinin-induced t-PA antigen and activity release were augmented two to threefold after typhoid vaccination in the absence of systemic hemodynamic effects (Fig. 2).

The underlying mechanisms for our findings are unknown. Inflammation is recognized to induce a protective response towards tissue injury, and it functions as part of normal host surveillance mechanisms. Various compounds associated with the inflammatory response, including histamine, thrombin and endotoxin, have been shown to increase cellular t-PA transcription and expression (33,34). However, we did not observe an increase in the basal plasma concentrations of either t-PA or PAI-1. The inflammatory stimulus, therefore, appears to augment specifically the storage or acute release of t-PA rather than a generalized upregulation of protein synthesis and basal secretion. This may be mediated by pro-inflammatory cytokines, such as IL-6, that modulate cellular activation, leading to alterations in endothelial function. In particular, molecular and pharmacologic evidence supports the role of bradykinin B2 receptors in the acute phase of inflammation, and upregulation of B2 receptors may account for the potentiation of bradykinin-induced t-PA release.

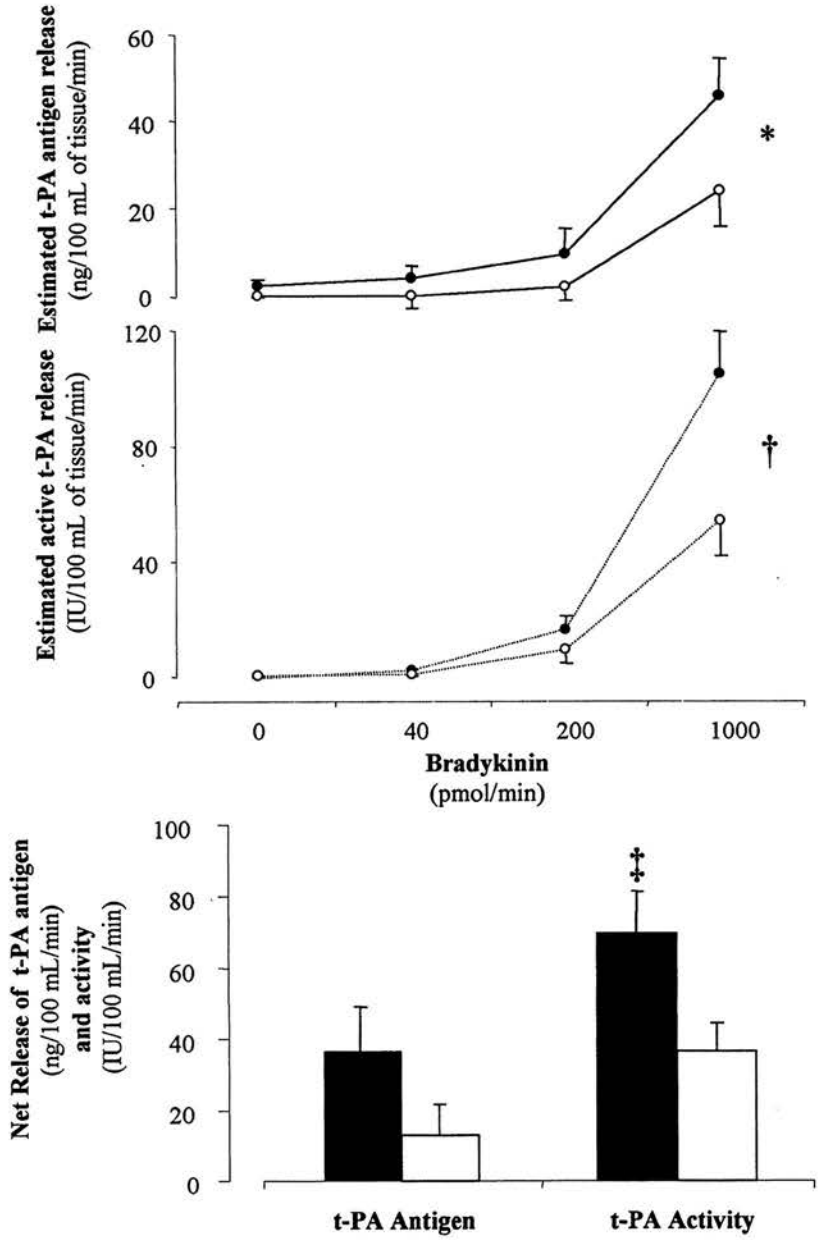

Figure 2. Estimated net release of tissue-type plasminogen activator (t-PA) antigen **(solid line)** and activity **(dotted line)** in subjects who were administered typhoid vaccination **(closed circles and bars)** and saline placebo **(open circles and bars). Lower panel** represents area under the curve for the response. Analysis of variance, *$p < 0.05$; †$p = 0.006$ (vaccination vs. placebo); *t* test, ‡$p < 0.05$ (vaccination vs. placebo).

Clinical implications. The augmentation of acute t-PA release after typhoid vaccination suggests that mild acute inflammation may induce antithrombotic properties in the forearm circulation. This may represent an adaptive response to inhibit intravascular thrombus deposition under circumstances of acute vascular inflammation. This observation is consistent with the increase in endogenous fibrinolysis in systemic inflammation induced by experimental endotoxemia in healthy subjects (35). However, in susceptible individuals, such as those with ischemic heart disease and chronic inflammation, this adaptive and protective acute response may fail or become depleted, leading to thrombus propagation and vessel occlusion. The fibrinolytic response to acute systemic inflammation in patients with ischemic heart disease and the influence of anti-inflammatory therapies, such as aspirin, now needs to be established. Indeed, recent evidence has suggested that preventative treatment with aspirin is able to reverse inflammation-induced endothelial dysfunction (36).

Epidemiologic studies have demonstrated an association between the risk of future cardiovascular events and both plasma inflammatory markers (11–13) and fibrinolytic factors (37). Therefore, the current observations are consistent with the suggestion that elevated plasma t-PA concentra-

tions may provide a marker of vascular inflammation. Irrespective of whether these common associations are partly or wholly explained by inflammation-induced t-PA release, understanding the regulation of both acute and chronic t-PA release will have important clinical implications and may help to develop more effective strategies in the management of atherosclerotic disease.

Study limitations. There are some limitations to our study. We administered the typhoid vaccination in the deltoid muscle of each subject's dominant arm rather than the gluteus muscle. However, because blood flow was assessed in the forearm and intra-arterial vasodilators were administered in the contralateral nondominant arm, it would be highly unlikely that the site of vaccination would have affected the response in the infused forearm. It would also have been preferable to assess the vascular responses immediately before and 6 to 8 h after vaccination on the same day. However, this would require repeated arterial cannulations within the same day, and we have previously demonstrated that endothelium-dependent vasodilation and t-PA release is reproducible when performed at least one week apart (38,39). The study subjects were healthy and young, and we acknowledge that the response in older subjects may be quite different. Finally, we studied peripheral vascular function; therefore, these findings may not be directly applicable to other vascular beds. However, endothelial dysfunction is often a generalized process, and we have previously shown (21,40) consistent endogenous fibrinolytic responses between the forearm and coronary circulation.

Conclusions. We have demonstrated that mild inflammation generated by typhoid vaccination results in a significant potentiation of bradykinin-induced t-PA release from the vascular endothelium. Additional studies are now required to determine the underlying mechanism and to assess the effects of acute and chronic inflammation on endogenous fibrinolysis in health and disease.

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Intra-Arterial Tumor Necrosis Factor-α Impairs Endothelium-Dependent Vasodilatation and Stimulates Local Tissue Plasminogen Activator Release in Humans

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Objective—Inflammation contributes to the pathogenesis of cardiovascular disease, potentially through the actions of proinflammatory cytokines. We assessed the direct effects of local intra-arterial tumor necrosis factor-α (TNF-α), interleukin-6, and endotoxin on blood flow and endogenous tissue plasminogen activator (t-PA) release in vivo in humans.

Methods and Results—In a double-blind, randomized, placebo-controlled trial, blood flow, plasma cytokine, and fibrinolytic parameters were measured using venous occlusion plethysmography and blood sampling. Ten subjects received intrabrachial TNF-α, interleukin-6, and endotoxin infusions, and 8 additional subjects received intrabrachial infusions of bradykinin, acetylcholine, and sodium nitroprusside after pretreatment with TNF-α. TNF-α but not interleukin-6, endotoxin, or placebo caused a gradual and sustained ≈20-fold increase in plasma t-PA concentrations ($P<0.001$) that was associated with elevated plasma interleukin-6 concentrations ($P<0.05$) but without an effect on blood flow or plasminogen activator inhibitor type 1 antigen. Compared with placebo, TNF-α pretreatment impaired bradykinin- and acetylcholine-induced vasodilatation ($P<0.03$) and resulted in a doubling of bradykinin-induced t-PA release ($P<0.05$).

Conclusions—Intra-arterial TNF-α causes an acute local vascular inflammation that is associated with impaired endothelium-dependent vasomotion as well as a sustained and substantial increase in endothelial t-PA release. TNF-α has potentially both adverse vasomotor and beneficial profibrinolytic effects on endothelial function. ***(Arterioscler Thromb Vasc Biol.* 2003;23:695-701.)**

Key Words: cytokines ■ endothelium ■ fibrinolysis ■ inflammation ■ vasodilatation

There is emerging evidence that systemic inflammation plays a major role in the pathogenesis of cardiovascular disease. The proinflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in particular, have been implicated in the initiation and maintenance of systemic and vascular inflammation associated with atherosclerosis and coronary artery disease. Indeed, plasma concentrations of these cytokines are elevated in patients with ischemic heart disease[1,2] and have been shown to predict the future risk of myocardial infarction in apparently healthy individuals.[3]

The vascular endothelium plays a vital role in the control of blood flow, hemostasis, fibrinolysis, and inflammation, and changes in endothelial function may underlie the association between inflammation and the risk of cardiovascular disease.

Tissue plasminogen activator (t-PA) is a fibrinolytic factor released from the endothelium and lyses intravascular fibrin.[4] If endogenous fibrinolysis is to be effective, then the rapid mobilization of t-PA from the endothelium is essential, because thrombus dissolution is much more effective if t-PA is incorporated during, rather than after, thrombus formation.[5] However, in the presence of proinflammatory states or an imbalance in endogenous fibrinolysis, intravascular thrombus may propagate, ultimately leading to arterial occlusion and tissue infarction.[6]

TNF-α and endotoxin have been reported to induce local vascular inflammation and impair endothelium-dependent vasodilatation in the venous circulation of humans.[7] Although mild systemic inflammation has also been shown to alter endothelial function,[8] the underlying mechanisms for these observations have not been elucidated, and the direct in vivo effects of cytokines and inflammatory stimuli on local arterial endothelial vasomotor and fibrinolytic function are unknown.

The aims of this study were to investigate the acute effects of local intra-arterial inflammatory cytokines (IL-6 and TNF-α) and bacterial endotoxin (lipopolysaccharide [LPS]) exposure on vasomotor function and endothelial t-PA release in vivo in humans.

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Methods

Subjects

Eighteen healthy nonsmoking men 21 to 25 years of age participated in the study, which was undertaken with the approval of the local research ethics committee in accordance with the Declaration of Helsinki and the written informed consent of each subject. None of the subjects had infective illnesses or received medication in the week before study. All subjects abstained from alcohol for 24 hours and from food and caffeine-containing drinks for at least 4 hours before each study. All studies were performed in a quiet, temperature-controlled room.

Cytokines and Drugs

TNF-α (Knoll Pharmaceuticals),[9] IL-6 (Novartis Pharma AG),[10] LPS (lot G-1, USPCI),[11] bradykinin (Clinalfa), acetylcholine (Cibavision Ophthalmics), and sodium nitroprusside (David Bull Laboratories) were administered after dissolution in 0.9% saline. TNF-α, IL-6, and LPS were prepared as stock solutions and stored at -80°C in aliquots. All other drugs were freshly prepared on the study day.

Hemodynamic Measurements

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges, as previously described.[12] Blood pressure was monitored in the noninfused arm at intervals throughout each study with a semiautomated noninvasive oscillometric sphygmomanometer.

Venous Sampling and Assays

Venous blood (10 mL) was withdrawn simultaneously from each arm and collected into tubes containing acidified buffered citrate (for t-PA assays), trisodium citrate (for plasminogen activator inhibitor type 1 [PAI-1] assays), and potassium ethylene diamine tetraacetic acid (EDTA) (for cytokine assays). Citrate and acidified buffered citrate samples were centrifuged at 2000*g* for 30 minutes at 4°C and EDTA samples at 1000*g* for 10 minutes at 20°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma t-PA, PAI-1, TNF-α, IL-6, and von Willebrand factor (vWF) antigen concentrations were determined as previously described[13,14] using enzyme-linked immunosorbent assays (Coaliza t-PA and PAI-1, Chromogenix AB; Quantikine human TNF and IL-6 immunoassays, R&D Systems; and Dako A/S, respectively) and fibrinolytic activities using a photometric method (Coatest t-PA and PAI-1, Chomogenix AB). Hematocrit and white cell count were determined using an automated Coulter counter (Beckman-Coulter ACt.8).

Study Design

Subjects rested recumbent, strain gauges and cuffs were applied, and venous cannulae (17-G) were inserted into both antecubital fossae. The brachial artery of the nondominant arm was cannulated with a 27-SWG needle under local anesthesia. The total rate of intra-arterial infusion was maintained constant throughout at 1 mL/min. Forearm blood flow was measured every 6 to 10 minutes.

Cytokine and Endotoxin Administration

In a randomized, double-blind study, 10 subjects attended on 3 occasions at least 1 week apart, and saline was infused for 30 minutes to allow time for equilibration. Subjects were then randomized to receive intra-arterial infusions of low-dose TNF-α (80 ng/min; n=6), high-dose TNF-α (240 ng/min; n=6), IL-6 (30 ng/min; n=6), LPS (100 pg/min; n=6), or saline placebo (1 mL/min; n=6) over 60 minutes. This was followed by an additional 60-minute saline washout infusion. Venous samples were obtained at baseline, 10, 20, 40, and 60 minutes during drug infusion, and 30, 60, and 180 minutes after cessation of drug infusion. Cytokine and LPS doses were chosen to achieve local concentrations comparable with healthy volunteer studies[7,11,15] and those seen in cardiovascular disease.[1,16]

Effect of Tumor Necrosis Factor-α on Endothelial Function

Eight subjects attended on 2 occasions at least 1 week apart in a randomized, double-blind, placebo-controlled, crossover trial. They received an intra-arterial infusion of either TNF-α (80 ng/min) or saline placebo (1 mL/min) over 60 minutes. After an additional 60 minutes of saline infusion, intra-arterial bradykinin was administered at 100, 300, and 1000 pmol/min for 10 minutes at each dose, acetylcholine at 5, 10, and 20 μg/min, and sodium nitroprusside at 2, 4, and 8 μg/min for 5 minutes at each dose. Infusions of the vasoactive drugs were separated by 15-minute infusions of saline. Venous samples were obtained at baseline, after 60 minutes of TNF-α/placebo infusion, before and during each dose of bradykinin, and 15 minutes after cessation of bradykinin infusion. Venous samples were not obtained during acetylcholine or sodium nitroprusside infusion, because they do not affect plasma t-PA or PAI-1 concentrations in this forearm model.[12,17,18]

Data Analysis and Statistics

Plethysmographic data were extracted from the chart data files, and the last 5 linear recordings in each measurement period were averaged. Estimated net t-PA antigen and activity release was defined previously[12–14] as the product of the infused forearm plasma flow and the concentration difference between the infused and noninfused forearms. Because basal t-PA concentrations were al-

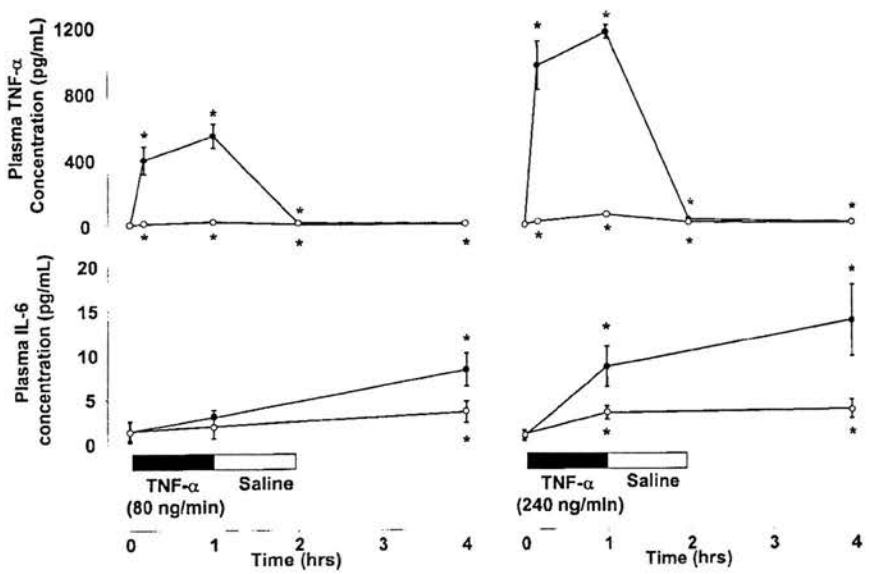

Figure 1. Plasma concentrations of TNF-α (top) and IL-6 (bottom) in the infused (●) and noninfused (○) arms after 1 hour of intra-arterial infusion of TNF-α at 80 ng/min (left) and 240 ng/min (right). $P \leq 0.01$ (ANOVA) for all responses except IL-6 concentrations in the noninfused arm. $P<0.05$ (ANOVA, infused vs noninfused arm) for all responses. *$P<0.05$ (paired *t* test; vs baseline).

TABLE 1. Forearm Blood Flow (mL/100 mL/min) During TNF-α, IL-6, LPS, and Saline Placebo Infusion

	TNF-α, 80 ng/min	TNF-α, 240 ng/min	IL-6, 30 ng/min	LPS, 100 pg/min	Saline
Infused arm					
Baseline	2.2±0.2	2.6±0.6	2.4±0.1	2.5±0.3	2.2±0.3
1 hour	1.9±0.3	2.4±0.9	1.9±0.2	2.3±0.3	2.0±0.3
2 hours	1.8±0.3	2.1±0.7	1.8±0.2	2.0±0.3	1.7±0.3
Noninfused arm					
Baseline	2.2±0.4	2.6±0.5	2.0±0.2	2.3±0.4	1.8±0.2
1 hour	2.6±0.5	2.6±0.8	2.0±0.3	2.0±0.3	1.7±0.2
2 hours	2.9±0.7	2.4±0.5	1.8±0.2	1.8±0.2	1.9±0.3

Values are mean±SEM.

tered by pretreatment with TNF-α, t-PA antigen and activity release during bradykinin infusion was calculated by subtracting the mean t-PA release before and 15 minutes after cessation of bradykinin infusion.

Data were examined, where appropriate, by ANOVA with repeated measures and 2-tailed paired Student's *t* test using Statview (SAS Institute, Inc). All results are expressed as mean±SEM. Statistical significance was assigned at the 5% level.

Results

All subjects remained well throughout the study and reported no adverse effects. There were no effects on hematocrit, body temperature, or white cell count throughout all studies (data on file).

Cytokine and Endotoxin Administration

Cytokine Assays

Plasma TNF-α concentrations increased from 1±0 and 2±1 pg/mL to 539±71 and 1164±41 pg/mL in the infused arm ($P<0.001$ for both) and to 20±2 and 62±8 pg/mL in the noninfused arm ($P<0.001$ for both) during 80 and 240 ng/min of TNF-α, respectively (Figure 1). This was accompanied by a gradual increase in plasma IL-6 concentrations (Figure 1). In the infused arm, IL-6 infusion increased plasma IL-6 concentrations from 2±1 to 14±3 pg/mL ($P=0.01$) and LPS infusion increased plasma TNF-α and IL-6 concentrations from 1±3 to 7±1 pg/mL ($P=0.01$) and from 1±0 to 6±2 pg/mL ($P=0.02$), respectively.

Hemodynamic Effects

Intra-arterial saline placebo, TNF-α, IL-6, and LPS infusions had no effect on heart rate, blood pressure, or forearm blood flow throughout all studies (data on file; Table 1).

Fibrinolytic and Hemostatic Assays

There were no changes in plasma t-PA antigen concentrations during IL-6, LPS, or saline placebo infusions (data on file). Plasma t-PA antigen and activity concentrations increased in the infused arm by up to 20-fold after both low- and high-dose TNF-α infusions ($P<0.001$; Figure 2). Plasma t-PA concentrations increased slowly, being detectable at 20 minutes and peaking at 60 minutes of infusion. Thereafter, plasma t-PA concentrations fell but remained elevated 4 hours after initiation of the infusion, with an apparently stable elevation between 2 and 4 hours.

Plasma PAI-1 and vWF antigen concentrations were unchanged throughout all studies, although plasma PAI-1 activity was reduced in the infused arm only after high-dose TNF-α infusion ($P=0.003$; Table 2).

Effect of Tumor Necrosis Factor-α on Endothelial Function

Cytokine Assays

Intra-arterial TNF-α infusion (80 ng/min) increased plasma TNF-α concentrations in the infused and noninfused arm from 2±1 and 2±1 pg/mL to 561±108 and 15±1 pg/mL, respectively ($P<0.001$ for both).

Hemodynamic Effects

Intra-arterial TNF-α or placebo infusion had no significant effects on heart rate, blood pressure, or basal forearm blood flow up to 2 hours after commencement of the infusion (P=NS for both). Forearm blood flow increased in a dose-dependent manner during bradykinin, acetylcholine, and so-

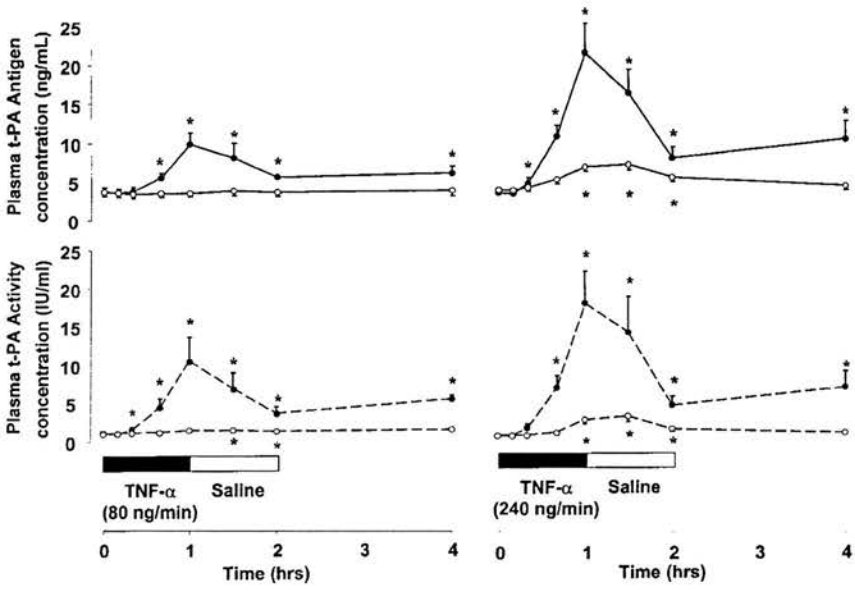

Figure 2. Plasma concentrations of t-PA antigen (solid lines) and activity (dashed lines) in the infused (●) and noninfused (○) arms after 1 hour of intra-arterial infusion of TNF-α at 80 ng/min (left) and 240 ng/min (right). $P<0.001$ (ANOVA) for all responses except noninfused arm after TNF-α 80 ng/min infusion. $P<0.001$ (ANOVA, infused vs noninfused arm) for all responses. *$P<0.05$ (paired *t* test; vs baseline).

TABLE 2. Plasma PAI-1 Antigen and Activity Concentrations During TNF-α Infusion

	TNF-α, 80 ng/min		TNF-α,240 ng/min	
	Infused	Noninfused	Infused	Noninfused
PAI-1 antigen, ng/mL				
0 minutes	17±4	19±5	23±5	24±5
60 minutes	18±4	20±4	25±6	26±8
120 minutes	14±3	16±3	18±5	24±5
240 minutes	15±4	14±3	21±5	36±17
PAI-1 activity, IU/mL				
0 minutes	12±2	11±1	10±2	9±2
60 minutes	9±2	10±2	5±2*	12±3
120 minutes	11±1	14±3	6±1*	10±2
240 minutes	8±1	11±2	4±1*	12±1

Values are mean±SEM.
*P=0.003, ANOVA, infused vs noninfused arm.

dium nitroprusside infusions ($P \leq 0.02$; Figure 3). Compared with placebo, TNF-α infusion caused impaired endothelium-dependent vasodilatation to bradykinin (P=0.029) and acetylcholine (P=0.01) but had no effect on endothelium-independent vasodilatation to sodium nitroprusside (P=0.43; Figure 3).

Fibrinolytic Assays

Plasma t-PA antigen and activity concentrations increased from 3.5±0.6 to 8.5±1.6 ng/mL (P=0.004) and from 1.1±0.3 to 9.2±1.9 IU/mL (P=0.003), respectively, after 60 minutes of TNF-α infusion. Again, this rise was sustained with an apparently stable elevation between 2 and 3 hours, ie, 1 to 2 hours after cessation of TNF infusion (Figure 4). Plasma t-PA antigen and activity concentrations were unchanged in the noninfused arm and during placebo infusion.

Plasma t-PA antigen and activity concentrations increased in a dose-dependent manner during bradykinin infusion ($P \leq 0.01$). Compared with placebo, pretreatment with TNF-α resulted in higher plasma t-PA antigen and activity concentrations during bradykinin infusion (11.3±0.8 versus 6.8±0.5 ng/mL and 16.5±3.9 versus 6.6±2.0 IU/mL at peak bradykinin dose; $P<0.002$) (Figure 4) and a marked augmentation in the estimated net release of t-PA antigen and activity (P=0.018 and P=0.037, respectively; Figure 5). For the 30-minute bradykinin dose-response infusion, pretreatment with TNF-α resulted in 83% and 132% increases in area under the curve for t-PA antigen and activity release, respectively ($P \leq 0.05$).

Discussion

We have demonstrated for the first time that local intra-arterial TNF-α administration can be used to generate an in vivo local vascular inflammatory model in humans that is associated with endothelial dysfunction and a unique profile of endogenous t-PA release. In contrast to IL-6 and LPS, TNF-α causes a slow-onset, sustained, and selective release of t-PA from the vascular endothelium in the absence of basal vasomotor effects. Moreover, pretreatment with TNF-α attenuates endothelium-dependent vasodilatation but augments

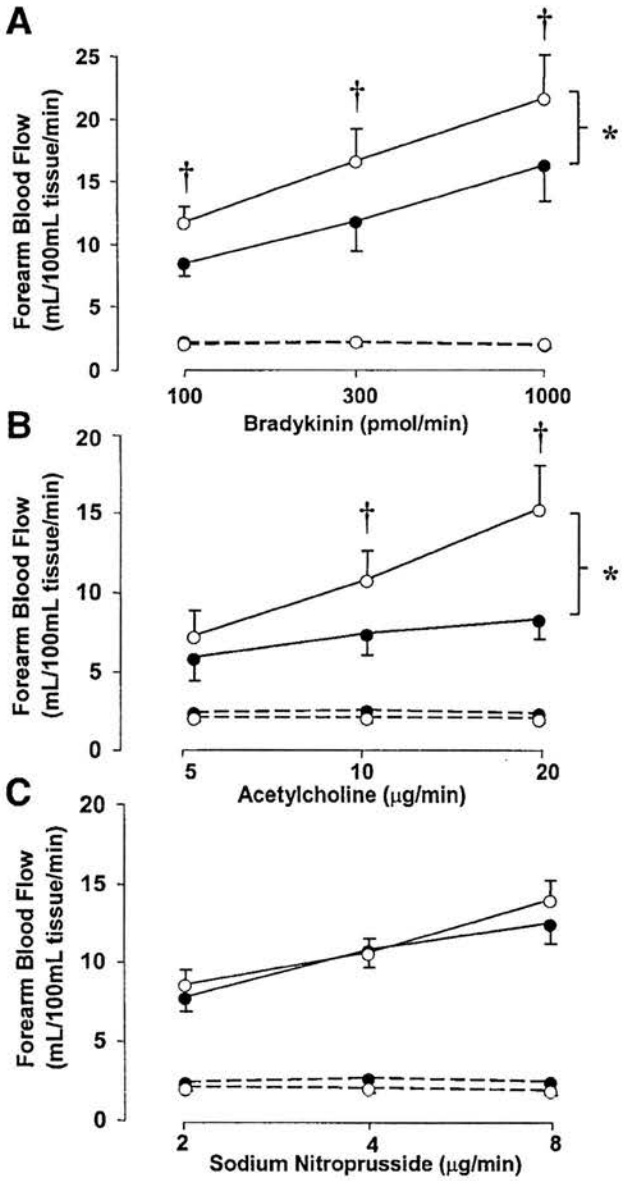

Figure 3. Infused (solid line) and noninfused (dashed line) forearm blood flow responses to incremental doses of bradykinin (a), acetylcholine (b), and sodium nitroprusside (c) in subjects who were pretreated with TNF-α (●) or saline placebo (○). $P \leq 0.02$ (ANOVA) for all infused arm responses. *$P<0.03$ (ANOVA, TNF-α vs saline placebo).

acute bradykinin-induced t-PA release. These findings indicate that TNF-α and acute vascular inflammation have complex effects on endothelial function. Although the profibrinolytic actions may reflect a protective mechanism in acute inflammation, TNF-α also directly impairs endothelium-dependent vasomotor responses.

Model of Local Vascular Inflammation

We have here developed a model of local vascular inflammation in vivo in humans. Using unilateral intrabrachial infusions, we achieved high local plasma TNF-α concentrations that were comparable to the plasma concentrations seen

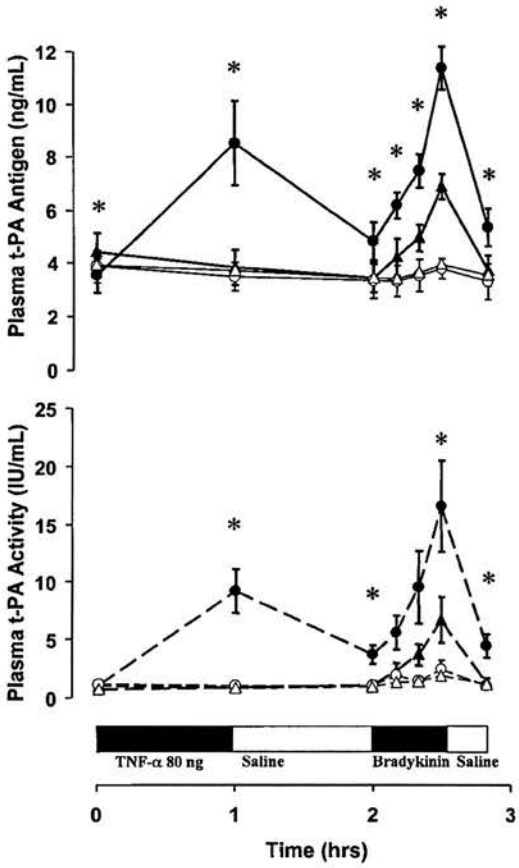

Figure 4. Plasma concentrations of t-PA antigen (solid lines) and activity (dashed lines) in infused (●) and noninfused (○) arms after 1 hour of intra-arterial infusion of TNF-α (circles) or saline placebo (triangles) and during bradykinin infusion. $P<0.001$ (ANOVA) for all infused arm responses. $P<0.001$ (ANOVA, infused versus noninfused arm) for all responses. $P<0.001$ (ANOVA, TNF-α vs saline placebo) for infused arm responses. *$P<0.05$ (paired t test; TNF-α vs placebo) for infused arm responses.

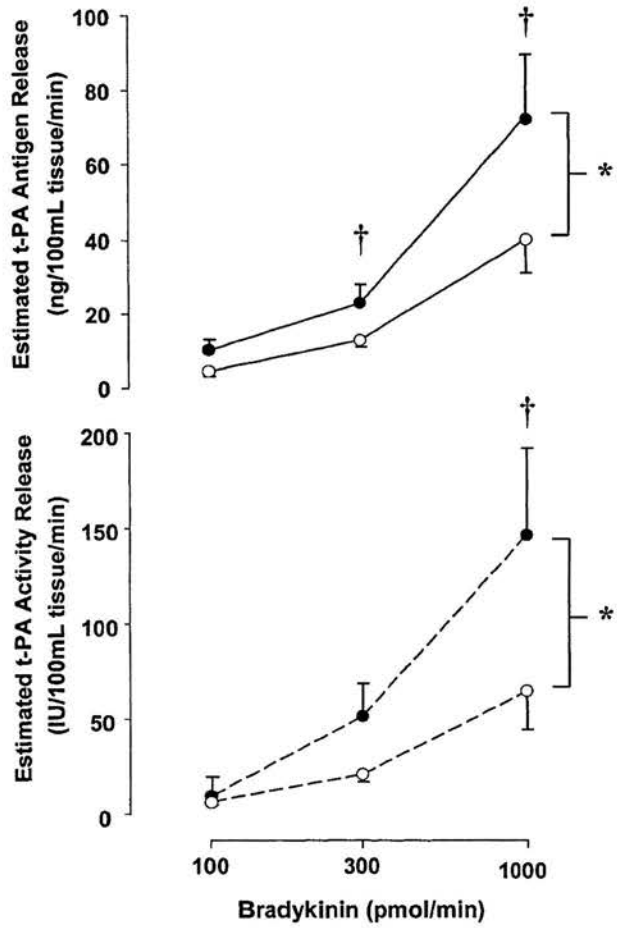

Figure 5. Estimated net t-PA antigen (solid lines) and activity (dashed lines) release during bradykinin infusion corrected for baseline t-PA concentrations in subjects who were pretreated with TNF-α (●) or saline placebo (○). $P<0.001$ (ANOVA) for all responses. *$P<0.05$ (ANOVA, TNF-α vs saline placebo). †$P\leq0.05$ (paired t test; TNF-α vs saline placebo).

in patients with severe heart failure.[16] A direct local vascular and endothelial inflammatory response was confirmed by the local rise in plasma IL-6 and t-PA concentrations. However, the fibrinolytic effects of TNF-α were not mediated through IL-6 release, because isolated IL-6 infusion had no effect on t-PA release. Indeed, in pilot studies, we found that high-dose intrabrachial IL-6 infusion sufficient to increase plasma IL-6 concentrations to >100 pg/mL have also failed to produce significant effects on forearm blood flow or t-PA release.

Effects of TNF-α on t-PA Release

The profile of t-PA release during local intra-arterial TNF-α administration is unique and has not been previously described in vivo in humans. Previous studies in healthy volunteers have reported changes in plasma fibrinolytic[15] and coagulation[20] factors during systemic TNF-α administration. TNF-α has pleiotropic effects and may cause these effects through actions on specific tissues or via secondary mediators released from organs such as the liver. In the present study, we have assessed local peripheral vascular responses to direct intra-arterial TNF-α and have shown that it causes selective endothelial t-PA release in the forearm without demonstrable effects on plasma vWF or PAI-1 antigen concentrations. Although there was a modest rise in the IL-6 and t-PA concentrations in the noninfused arm, this may represent overspill from the infused arm, where the concentrations increased by up to 20-fold. In contrast to previous systemic studies,[15,20] subjects here remained asymptomatic and there was no associated pyrexia, consistent with the absence of a major systemic response.

We[12] and others[17,18] have previously reported acute rapid t-PA release during intra-arterial substance P, bradykinin, and methacholine infusions. Using these agents, there is a near-instantaneous onset and offset of action with no sustained increase in t-PA release after cessation of administration. Moreover, there is always an associated change in vascular tone and regional blood flow, because these agents also cause vasodilatation. In contrast, TNF-α administration had no effect on basal blood flow and caused a slow onset and sustained release of t-PA that was not apparent until 20

Endothelial dysfunction in patients with recent myocardial infarction and hyperhomocysteinaemia: effects of vitamin supplementation

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ABSTRACT

Hyperhomocysteinaemia is a prothrombotic condition that may cause oxidative endothelial injury and impair endogenous fibrinolysis. Vitamin supplementation enhances endothelial function in hyperhomocysteinaemic patients, but responses in patients with co-existing coronary artery disease have been variable. It is also unknown whether hyperhomocysteinaemia is associated with reduced fibrinolytic responses in patients with coronary artery disease. The study aims were to test the hypothesis that patients with recent myocardial infarction and hyperhomocysteinaemia have impaired endothelium-dependent vasomotion and fibrinolysis that is rectified by vitamin supplementation. From a cohort of 120 patients admitted with acute myocardial infarction, 18 patients were recruited from the upper ($n = 9$) and lower ($n = 9$) plasma homocysteine quartiles into a randomized double-blind placebo-controlled crossover trial. Following a 4-week course of placebo or folate/cyanocobalamin/pyridoxine supplements, FBF (forearm blood flow) was measured using venous occlusion plethysmography during intra-arterial substance P (4–16 pmol/min), acetylcholine (5–20 μg/min) and sodium nitroprusside (2–8 μg/min) infusions. All vasodilators caused dose-dependent increases in infused FBF ($P < 0.05$). Patients in the upper homocysteine quartile (16.8 ± 2.9 compared with 7.9 ± 0.7 μmol/l; $P = 0.003$) had reduced vasodilatation to acetylcholine ($P = 0.01$) and substance P ($P < 0.05$), but not sodium nitroprusside. There were no differences in substance P-induced tissue plasminogen activator release. Vitamin supplementation increased serum folate and vitamin B_{12} concentrations ($P < 0.05$), but did not significantly lower homocysteine, or affect FBF or fibrinolytic responses. In patients with recent myocardial infarction, hyperhomocysteinaemia is associated with impaired endothelium-dependent vasodilatation, but no alteration in the acute fibrinolytic capacity. This endothelial vasomotor dysfunction is unaltered by vitamin supplementation.

INTRODUCTION

Several prospective and case-control studies have shown that elevated plasma homocysteine concentrations are an independent risk factor for the development of atherothrombotic vascular disease as well as a prognostic marker in ischaemic heart disease [1–3]. Plasma homocysteine concentrations are consistently higher in patients with premature peripheral and cerebrovascular diseases [4], and almost a third of patients with premature coronary

Key words: endothelial dysfunction, fibrinolysis, homocysteine, myocardial infarction, vasodilatation, vitamin.

Abbreviations: CI, confidence interval; FBF, forearm blood flow; NS, not significant; PAI-1, plasminogen activator inhibitor type 1; t-PA, tissue plasminogen activator.

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artery disease are found to have hyperhomocysteinaemia [1]. In addition, apparently healthy men with plasma homocysteine concentrations 12 % above the upper limit of normal have a 3-fold increased risk of acute myocardial infarction [2], and in patients with ischaemic heart disease there is an increased mortality associated with plasma concentrations greater than 9 μmol/l [5].

The vascular endothelium plays a central role in the control of blood flow, haemostasis and endogenous fibrinolysis, and endothelial dysfunction independently predicts cardiovascular events [6,7]. Although the mechanism of vascular damage is unclear, homocysteine may promote atherogenesis through oxidative endothelial injury that is mediated by cytotoxic reactive oxygen species [8–10]. Indeed, acute and chronic hyperhomocysteinaemia are associated with impaired endothelium-dependent flow-mediated dilatation of the brachial artery [10–12].

Hyperhomocysteinaemia is a prothrombotic state [3]. We [13] and others [14] have shown previously that hyperhomocysteinaemia induced by oral methionine loading is associated with alterations in endogenous fibrinolysis in healthy subjects and patients with premature vascular disease. However, the influence of chronic hyperhomocysteinaemia on the acute fibrinolytic capacity is unknown and is the subject of debate [15]. Interestingly, there is an association between plasma homocysteine and t-PA (tissue plasminogen activator) antigen concentrations in stroke patients [16].

Vitamin supplementation with folate, vitamin B_6 and vitamin B_{12} is safe and may reduce plasma homocysteine concentrations [17]. Although endothelial function is enhanced following treatment with folate in patients with hyperhomocysteinaemia [18,19] and hypercholesterolaemia [20], the response in patients with coronary artery disease has been variable [21–25]. Furthermore, it is unknown whether elevated plasma homocysteine concentrations are associated with reduced resistance vessel vasomotor responses in patients with established coronary artery disease.

The aim of the present study was to test the hypotheses that, in patients with recent myocardial infarction, elevated plasma homocysteine concentrations are associated with impaired endothelium-dependent vasodilatation and endogenous fibrinolytic capacity, and that vitamin supplementation (with folate, vitamin B_6 and vitamin B_{12}) would both lower plasma homocysteine and restore endothelial function.

METHODS

Subject recruitment

One hundred and twenty patients admitted with an acute myocardial infarction were recruited into the trial. Myocardial infarction was defined as typical ischaemic cardiac pain associated with elevation of cardiac markers (greater than twice the upper limit of normal) and electrocardiographic evidence of myocardial ischaemia. Exclusion criteria were atrial fibrillation on warfarin therapy, impaired renal function (serum creatinine >120 μmol/l), diabetes mellitus, requirement for folate supplementation or pernicious anaemia. The written informed consent of each subject was obtained before entry into the study. All studies were undertaken with the approval of the local Research Ethics Committee and in accordance with the Declaration of Helsinki (1996).

Study design

Fasting plasma homocysteine concentrations were determined in all patients on days 5–7 following acute myocardial infarction [26]. From the upper and lower plasma homocysteine concentration quartiles, nine patients in each quartile were recruited into a randomized double-blind balanced-block placebo-controlled crossover trial at least 4 months after the index event. All patients received two separate 4-week courses of oral sucrose placebo or vitamin supplementation (5 mg of folate/100 μg of cyanocobalamin/10 mg of pyridoxine), and attended at the end of each 4-week treatment period. On each study day, medications were withheld and subjects attended after a 4-h fast and rested recumbent in a quiet temperature-controlled room maintained at 22–25 °C. Strain gauges and cuffs were applied, and the brachial artery of the non-dominant arm was cannulated. After 30 min equilibration with saline infusion, intra-arterial substance P (4–16 pmol/min), acetylcholine (5–20 μg/min) and sodium nitroprusside (2–8 μg/min) were administered in a randomized order for 6–10 min at each dose and separated by 20 min washout periods [27–29]. Venous samples were taken at baseline and during infusion of each substance P dose for determination of t-PA and PAI-1 (plasminogen activator inhibitor type 1). Venous sampling was not performed during sodium nitroprusside or acetylcholine infusion, since they do not affect t-PA or PAI-1 release in this forearm model [13,27,30].

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, U.K.) under 1 % lidocaine (Xylocaine; Astra Pharmaceuticals, Kings Langley, Herts., U.K.) local anaesthesia and attached to a 16-gauge epidural catheter (Portex, Hythe, Kent, U.K.). Patency was maintained by infusion of saline (0.9 % NaCl) via a MS2000 syringe infusion pump (Graseby Medical, Watford, Herts., U.K.). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 ml/min. Pharmaceutical grade substance P (Clinalfa, Läufelfingen, Switzerland), acetylcholine (Cibavision Ophthalmics, Southampton, U.K.) and sodium nitroprusside (David Bull Laboratories, Warwick,

U.K.) were administered following dissolution in saline. All solutions were freshly prepared on the day of study.

Haemodynamic measurements

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges as described previously [27,28]. During measurement periods, the hands were excluded from the circulation by rapid inflation of the wrist cuffs to 220 mmHg using E20 rapid cuff inflators (DE Hokanson, Bellevue, WA, U.S.A.). Upper arm cuffs were inflated intermittently to 40 mmHg for 8 s in every 10 s to achieve venous occlusion and obtain plethysmographic recordings. Blood pressure was monitored in the non-infused arm at intervals throughout each study with a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751; Takeda Medical, Tokyo, Japan).

Venous sampling and assays

Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms. Venous blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool® Stabilyte™, Umeå, Sweden) for t-PA assays and trisodium citrate and lithium heparin tubes (Monovette®, Sarstedt, Nümbrecht, Germany) for PAI-1 and homocysteine assays respectively. Samples were kept on ice before being centrifuged at 2000 *g*. Platelet-free plasma was decanted and stored at $-80\,^{\circ}C$ before assay. Plasma t-PA and PAI-1 antigens were determined as described previously [27] using ELISA (Coaliza t-PA and Coaliza PAI-1; Chromogenix, Mölndal, Sweden), and plasma t-PA activity was determined using a photometric method (Coatest t-PA; Chromogenix). Plasma homocysteine (Axis® Homocysteine EIA; Axis-Shield, Oslo, Norway) and serum folate and vitamin B_{12} concentrations (Bayer Immuno 1® automated immunoassay analyser, Bayer, Leverkusen, Germany) were determined using enzyme immunoassay.

Data analysis and statistics

Estimated net release of t-PA antigen and activity were defined previously [27] as the product of the infused forearm plasma flow [based on the mean haematocrit (Hct) and the infused FBF (forearm blood flow)] and the concentration difference between the infused ($[t\text{-}PA]_{inf}$) and non-infused ($[t\text{-}PA]_{Noninf}$) arms using the formula:

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

Data were examined, where appropriate, by ANOVA with repeated measures and two-tailed paired Student's *t* test (SAS Institute). All results are expressed as means (S.E.M.) or means [95% CI (confidence interval)]. Statistical significance was taken at the 5% level.

Table 1 Patient characteristics

Values are means (S.E.M.), or numbers (percentage). ACE, angiotensin-converting enzyme.

	Patients in upper quartile	Patients in lower quartile
n	9	9
Age (years)	54 (2)	57 (5)
Sex (male/female)	9/0	8/1
Mean arterial pressure (mmHg)	88 (5)	86 (2)
Heart rate (beats/min)	59 (2)	54 (2)
Type of myocardial infarction		
Anterior (*n*)	2 (22%)	3 (33%)
Inferior (*n*)	6 (67%)	3 (33%)
Other (*n*)	1 (11%)	3 (33%)
Peak creatine kinase (units/l)	790 (230)	1900 (550)
Received thrombolysis (*n*)	6 (67%)	6 (67%)
Medication		
Antiplatelet therapy (*n*)	9 (100%)	9 (100%)
β-Adrenoceptor antagonists (*n*)	8 (89%)	6 (67%)
ACE inhibitors (*n*)	5 (56%)	5 (56%)
HMG CoA reductase inhibitors (*n*)	8 (89%)	7 (77%)
Risk factors		
Smokers (*n*)	6 (67%)	3 (33%)
History of hypertension (*n*)	3 (33%)	2 (22%)
Hypercholesterolaemia (*n*)	8 (89%)	7 (77%)
Diabetes mellitus (*n*)	0 (0%)	0 (0%)

RESULTS

Baseline and biochemical characteristics

From the upper and lower plasma homocysteine quartiles, 18 patients (nine patients in each quartile) were recruited into the randomized controlled trial. Apart from plasma homocysteine concentrations ($P < 0.01$, as determined by Student's *t* test), which were significantly different by design, there were no significant differences between the baseline clinical characteristics or medical therapies in the two groups of patients (Tables 1 and 2).

All subjects tolerated placebo and vitamin supplementation and no side effects were reported or noted. Serum folate and vitamin B_{12} concentrations were increased following vitamin supplementation compared with placebo in both patient groups ($P < 0.001$ and $P < 0.05$ respectively, as determined by Student's *t* test; Table 2). Plasma homocysteine concentrations appeared to be reduced by $\approx 16\%$ with active treatment in hyperhomocysteinaemic patients, but this was not statistically significant ($P = 0.3$, as determined by Student's *t* test; Table 2). Vitamin supplementation had no significant effects on heart rate, blood pressure or basal FBF in either patient group.

Table 2 Plasma homocysteine, serum folate and vitamin B_{12} concentrations following vitamin supplementation

Values are means (S.E.M.). *$P < 0.01$ compared with lower quartile, as determined by Student's t test; †$P < 0.05$ compared with placebo, as determined by Student's t test.

	Patients in the upper quartile		Patients in the lower quartile	
	Placebo	Treatment	Placebo	Treatment
Plasma homocysteine (μmol/l)	16.8 (2.8)*	14.2 (1.1)*	7.9 (0.7)	7.8 (0.7)
Serum folate (μg/l)‡	8.6 (1.8)	17.7 (1.6)†	9.0 (1.2)	19.9 (0.1)†
Serum vitamin B_{12} (ng/l)	462 (136)	580 (149)†	380 (39)	431 (48)†

‡ Upper limit of serum folate assay was 20 μg/l; 13 patients had concentrations above this after supplementation and were taken as 20 μg/l.

Endothelium-dependent vasomotion

Substance P, acetylcholine and sodium nitroprusside caused dose-dependent increases in blood flow in the infused forearm in both patient groups on each study day ($P < 0.05$, as determined by ANOVA; Figure 1). In comparison with patients in the lower quartile, hyperhomocysteinaemic patients had significantly reduced FBF responses to acetylcholine and substance P ($P = 0.01$ and $P < 0.05$ respectively, as determined by ANOVA; Figure 1), but there were no significant differences in the blood flow responses to sodium nitroprusside [P = NS (not significant), as determined by ANOVA].

Neither endothelium-dependent nor endothelium-independent vasodilatation were significantly influenced by vitamin treatment in either patient group (P = NS for all, as determined by ANOVA). For the hyperhomocysteinaemic group, the mean difference for the response to vitamin treatment at the peak dose was 1.1 ml · 100 ml^{-1} · min^{-1} (95% CI, −0.8 to +2.9) for substance P, 0.3 ml · 100 ml^{-1} · min^{-1} (95% CI, −1.3 to +1.8) for acetylcholine, and −0.3 ml · 100 ml^{-1} · min^{-1} (95% CI, −2.8 to +2.1) for sodium nitroprusside. For the lower quartile group, the mean difference for the response to vitamin treatment at the peak dose was 2.0 ml · 100 ml^{-1} · min^{-1} (95% CI, −0.6 to +4.6) for substance P, −0.8 ml · 100 ml^{-1} · min^{-1} (95% CI, −2.9 to +1.2) for acetylcholine, and 1.7 ml · 100 ml^{-1} · min^{-1} (95% CI, −2.8 to +6.2) for sodium nitroprusside.

Fibrinolytic responses

Baseline plasma t-PA and PAI-1 antigen and t-PA activity concentrations were similar in both groups (Table 3). Substance P caused dose-dependent increases in plasma t-PA antigen and activity concentrations in the infused forearm of all patients ($P < 0.05$ for both, as determined by ANOVA; Table 3). The responses were similar in both patient groups and were not influenced by vitamin supplementation (P = NS, as determined by ANOVA). The mean difference of t-PA antigen release for the response to vitamin supplementation at the peak substance P dose was −2.7 ng · 100 ml^{-1} · min^{-1} (95% CI, −15.5 to +10.2; P = NS, as determined by Student's t test) in the upper quartile group and −7.4 ng · 100 ml^{-1} · min^{-1} (95% CI, −24.3 to +9.5, P = NS, as determined by Student's t) in the lower quartile group. Plasma PAI-1 concentrations were unaffected by substance P infusion or vitamin treatment (P = NS, as determined by Student's t test).

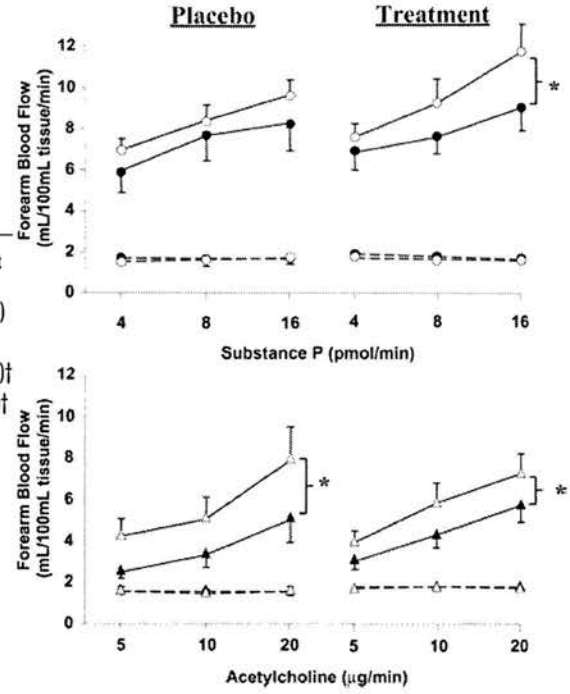

Figure 1 Infused (solid line) and non-infused (dashed line) FBF responses to incremental doses of substance P (upper panel) and acetylcholine (lower panel) in patients in upper (closed symbols) and lower quartiles (open symbols) following placebo (left panel) and vitamin supplementation (right panel)

$P < 0.05$ for all infused arm responses, as determined by ANOVA. *$P < 0.05$ compared with lower quartile, as determined by ANOVA.

DISCUSSION

In the present study, we have demonstrated that, in patients with a recent myocardial infarction, elevated plasma homocysteine concentrations are associated with impaired endothelium-dependent vasodilatation without affecting acute endogenous t-PA release. However, vitamin supplementation failed to significantly reduce plasma homocysteine concentrations or improve endothelial vasomotor function.

Table 3 Plasma t-PA and PAI-1 concentrations and release during substance P infusion

Values are means (S.E.M.). $*P < 0.05$ for all responses during substance P infusion, as determined by ANOVA. AUC, area under curve; IU, international units.

		Patients in the upper quartile				Patients in the lower quartile			
		Placebo		Treatment		Placebo		Treatment	
	Substance P (pmol/min)	Infused arm	Non-infused arm	Infused arm	Non-infused arm	Infused arm	Non-infused arm	Infused arm	Non-infused arm
t-PA antigen (ng/ml)	Baseline	7.7 (1.2)	8.6 (1.4)	7.5 (1.0)	8.2 (1.4)	6.6 (1.2)	6.5 (1.3)	7.2 (1.3)	7.6 (1.3)
	4	9.8 (1.5)	8.3 (1.3)	9.2 (1.0)	7.7 (1.3)	7.6 (0.9)	7.0 (1.1)	8.1 (1.4)	7.6 (1.4)
	8	9.4 (1.5)	8.1 (1.1)	10.3 (1.1)	7.8 (1.0)	9.3 (1.4)	7.1 (1.2)	8.9 (1.5)	7.6 (1.5)
	16	12.7 (2.4)*	7.8 (0.9)	11.1 (1.4)*	7.6 (1.1)	10.6 (1.2)*	6.7 (1.3)	10.5 (1.6)	7.9 (1.4)
Estimated t-PA antigen release ($ng \cdot 100\ ml^{-1} \cdot min^{-1}$)	Baseline	− 1.1 (0.7)		− 1.2 (1.1)		0.4 (0.7)		− 0.3 (0.4)	
	4	4.0 (3.2)		5.4 (3.4)		3.4 (2.2)		1.4 (1.5)	
	8	4.7 (4.1)		11.1 (4.2)		9.8 (3.7)		5.9 (2.4)	
	16	21.6 (6.7)*		18.9 (5.2)*		24.3 (8.4)*		15.6 (3.9)*	
Net t-PA antigen release (AUC)		18.9 (9.3)		25.4 (9.0)		24.2 (8.5)		14.9 (4.2)	
t-PA activity (IU/ml)	Baseline	0.9 (0.2)	0.8 (0.2)	0.5 (0.1)	0.6 (0.1)	1.0 (0.3)	1.0 (0.3)	0.8 (0.2)	0.8 (0.3)
	4	1.7 (0.6)	0.7 (0.2)	1.5 (0.3)	0.8 (0.1)	2.5 (0.8)	1.2 (0.4)	1.5 (0.6)	0.9 (0.2)
	8	1.9 (0.7)	0.8 (0.3)	2.3 (0.4)	0.7 (0.2)	2.8 (0.9)	1.2 (0.4)	2.0 (0.9)	0.9 (0.2)
	16	3.1 (0.8)*	1.0 (0.2)	2.9 (0.5)*	0.8 (0.2)	3.9 (1.1)*	1.5 (0.4)	2.7 (1.0)*	0.9 (0.2)
Estimated t-PA activity release ($IU \cdot 100\ ml^{-1} \cdot min^{-1}$)	Baseline	0.0 (0.1)		− 0.1 (0.1)		0.0 (0.1)		0.1 (0.2)	
	4	2.5 (1.4)		3.1 (1.3)		6.0 (2.1)		2.2 (1.2)	
	8	3.6 (1.6)		7.0 (1.7)		8.4 (3.6)		4.6 (2.1)	
	16	9.3 (2.9)*		11.7 (3.6)*		16.4 (6.9)*		10.2 (3.4)*	
Net t-PA activity release (AUC)		10.7 (4.2)		15.9 (4.7)		21.6 (8.7)		12.0 (4.9)	
PAI-1 antigen (ng/ml)	Baseline	47 (5)	48 (4)	44 (4)	48 (5)	45 (14)	46 (14)	44 (11)	46 (13)
	16	48 (4)	54 (3)	45 (4)	50 (5)	44 (15)	44 (15)	46 (12)	49 (15)

Endothelium-dependent vasodilatation

Previous studies have shown that homocysteine is associated with endothelial dysfunction. Lentz et al. [9] reported that, in monkeys with diet-induced hyperhomocysteinaemia, endothelium-dependent vasodilatation is impaired in carotid artery rings *in vitro* and hindlimb resistance vessels *in vivo*. Celermajer et al. [11] documented abnormal endothelium-dependent vasodilatation in children with severe hyperhomocysteinaemia due to homozygous homocystinuria. Impaired endothelium-dependent vasodilatation is detected in healthy subjects with acute hyperhomocysteinaemia induced by oral methionine loading [10,31], as well as in patients with chronic hyperhomocysteinaemia who are free from clinical manifestations of atherosclerotic disease [12,18]. In the present study, we have extended these findings to patients with recent myocardial infarction and demonstrated impaired vasomotor responses in those with elevated plasma homocysteine concentrations.

The vascular endothelium plays a critical role in the control of vascular homoeostasis by regulating vascular tone, platelet activity, coagulation and fibrinolysis, and endothelial dysfunction is believed to be an early step in the pathogenesis and pathophysiology of atherosclerosis. Although patients with coronary artery disease typically demonstrate endothelial dysfunction, there is considerable heterogeneity in the magnitude of impairment in individuals with similar risk factor profiles. This is of particular interest because the extent of coronary as well as peripheral endothelial dysfunction independently predicts the long-term risk of acute cardiovascular events, including sudden cardiac death, myocardial infarction and revascularization procedures [6,7]. Recent prospective data have indicated that, in patients with established coronary artery disease, homocysteine is a significant predictor of mortality independent of other traditional risk factors [5,32]. Our present findings therefore support the role of homocysteine as a secondary risk marker, suggesting that this may be mediated through its effects on endothelial function.

Endogenous fibrinolysis

Although homocysteine impairs endothelial vasomotor function, it does not appear to have a major effect on endothelium-dependent fibrinolytic capacity, as both basal and stimulated release of t-PA or PAI-1 were not significantly different between the two patient groups. Hyperhomocysteinaemia is a prothrombotic condition

and may interfere with the antithrombotic and fibrinolytic mechanisms of the endothelium and alter endothelial protein secretory pathways. Although endothelial cell-associated t-PA activity is reduced in homocysteine-treated cells [33], our present study failed to detect reduced fibrinolytic activity *in vivo*. This is consistent with data indicating that homocysteine might perturb the intrinsic fibrinolytic potential by reducing the functional binding site for t-PA without altering the catalytic capability of t-PA or affecting t-PA synthesis and secretion [33].

Effects of vitamin supplementation on plasma homocysteine

Previous studies have shown that treatment with folate and B vitamins can lower plasma homocysteine concentrations to a varying degree. The Homocysteine Lowering Trialists' meta-analysis predicted a 20–30 % reduction in homocysteine in patients with plasma concentrations above 12 μmol/l taking folate, and a further small additional effect with vitamin B_{12} but not B_6 [17]. However, a more modest 11–14 % reduction is seen in patients with coronary artery disease who consumed fortified breakfast cereals [34,35]. The limited homocysteine lowering seen in our present study was probably related to the relatively mild hyperhomocysteinaemia and normal folate concentrations in our study population as well as the confounding effects of dietary folate fortification.

Effects of vitamin supplementation on endothelial responses

We did not detect an improvement in endothelium-dependent vasodilatation or endogenous fibrinolytic capacity following vitamin supplementation in the present study. Although this may not be surprising in the absence of significant homocysteine reduction, Doshi et al. [22] have suggested that the acute effects of folate on endothelial function may occur by a mechanism independent of homocysteine lowering. Moreover, the evidence of the beneficial effects of vitamin supplementation on endothelial function is conflicting. Chambers et al. [21] and Title et al. [24] demonstrated improved endothelial function in patients with coronary artery disease following folate treatment without or with vitamin B_{12}. However, our present findings are consistent with those of other investigators who failed to detect improved endothelial function in a similar patient population, in healthy siblings of patients with premature atherothrombotic disease or in patients with renal impairment [23,25,36]. These contradictory findings may be related, in part, to the presence of other cardiovascular risk factors such as hypertension or hypercholesterolaemia, which may contribute to endothelial injury [37,38], but would not be expected to respond to folate or B vitamins. The vascular endothelium in patients with established coronary artery disease may have also been subjected to chronic injury and would therefore be less responsive to intervention. Furthermore, although the above studies adopted flow-mediated dilatation as a non-invasive method of assessing conduit artery endothelial function, in the present and other studies [28,39], we have focussed on the function of endothelium within resistance vessels. Conduit artery and microvascular endothelial cells have distinct phenotypic differences, and responses to mechanical rather than pharmacological stimuli may also differ, and may contribute to the apparent disparity in the responses.

There is further controversy regarding the effects of vitamin supplementation on cardiovascular outcomes in patients undergoing percutaneous coronary intervention. Contrary to earlier reports that vitamin supplementation may reduce the rate of restenosis and adverse outcomes following coronary artery angioplasty [40,41], Lange et al. [42] have recently demonstrated that folate therapy following coronary stenting may increase the risk of in-stent restenosis. The underlying mechanism for these findings remains uncertain, but it is possible that the proliferative effects of folate may promote the growth of neointimal cells within implanted stents. Therefore more prospective data are needed before any recommendations can be made regarding the use of vitamin supplementation in coronary artery disease.

Study limitations

There are several potential limitations to our present study. First, we studied peripheral vascular function and thus these findings may not be directly applicable to other vascular beds. However, endothelial dysfunction is often a generalized process, and we have shown previously consistent vasomotor and endogenous fibrinolytic responses between the forearm and coronary circulation [27,39]. Secondly, the failure to improve endothelial function may be related to inadequate treatment duration, although studies have suggested that a 4–6-week treatment with folate can improve endothelial function [20,22]. Thirdly, the size of the study was small and, although it was powered to detect a 15–20 % difference in t-PA release or forearm vasodilatation, it is possible that a smaller effect may have been missed. Fourthly, we used a placebo-controlled crossover design and there was the possibility of a carry-over effect of the vitamin therapy on endothelial function during placebo administration, despite a significant difference in the serum concentrations of folate and vitamin B_{12}. Finally, we cannot rule out that homocysteine may be a marker of vascular injury rather than a mediator of endothelial dysfunction, although experimental data support the direct role of homocysteine in causing endothelial damage [9,33].

In conclusion, we have demonstrated that endothelium-dependent vasodilatation, but not endogenous

fibrinolysis, is impaired in patients with recent myocardial infarction and elevated plasma homocysteine, and that this endothelial vasomotor dysfunction is not rectified by vitamin supplementation. These results provide further evidence for the role of homocysteine in vascular damage, but do not support the hypothesis that vitamin supplementation improves endothelial function in patients with established coronary artery disease.

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Regular Article

Preserved endothelial vasomotion and fibrinolytic function in patients with acute stent thrombosis or in-stent restenosis

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Abstract

Introduction: Acute stent thrombosis and in-stent restenosis are serious complications of percutaneous coronary intervention (PCI) and may be associated with vascular or platelet abnormalities. We aimed to assess endothelium-dependent vasomotion, endogenous fibrinolysis and platelet function in patients with acute stent thrombosis or in-stent restenosis. *Materials and methods*: Thirty-six subjects were enrolled into four groups: acute stent thrombosis, in-stent restenosis, uncomplicated PCI with stent implantation and healthy matched controls. Forearm blood flow was measured using bilateral venous occlusion plethysmography during intra-brachial acetylcholine, substance P and sodium nitroprusside infusion. Venous blood samples were withdrawn for estimation of plasma fibrinolytic variables and platelet aggregometry. *Results*: Acetylcholine, substance P and sodium nitroprusside caused dose-dependent increases in blood flow ($P<0.001$) and substance P caused a dose-dependent increase in tissue-type plasminogen activator (t-PA) release ($P<0.001$) in all groups. Thrombin, collagen, adenosine diphosphate (ADP) and the thromboxane A2 analogue, U46619, caused dose-dependent platelet aggregation ($P<0.001$) in all groups. There were no significant between group differences in these responses except that, in keeping with aspirin therapy, collagen-induced platelet aggregation was impaired in patient groups compared with healthy controls ($P<0.01$). Post-hoc analysis demonstrated a significant impairment of acute t-PA release in current smokers compared to non-smokers ($P<0.05$). *Conclusions*: Despite previous reports suggesting impaired vascular function, endothelium-dependent vasomotion, endogenous fibrinolysis and platelet aggregation do not appear to play a major role in the pathogenesis of acute stent thrombosis or in-stent restenosis.

Keywords: Endothelial function; Platelets; In-stent restenosis; Stent thrombosis; Fibrinolysis

Coronary artery stent implantation is a valuable adjunct to percutaneous transluminal coronary angioplasty. It reduces the absolute incidence of restenosis compared with balloon angioplasty by 10–15% and improves 6-month event free survival by 10–20% [1,2]. However, there is a small but significant risk of acute coronary stent thrombosis [3] and in-stent restenosis that can have devastating consequences including myocardial infarction and death [4]. Whilst procedure-related complications such as persistent dissection, longer stent length and final minimal lumen diameter may be implicated in some cases of stent thrombosis or restenosis [3], no underlying precipitant can be identified in many patients.

Stent thrombus formation is principally initiated by platelet aggregation, which, in the absence of effective endothelium-derived vasoregulation and fibrinolysis, is then stabilised by the deposition of a fibrin mesh. The chronology of in-stent restenosis has been described as early thrombosis, followed by thrombus endothelialization and infiltration by lymphocytes and monocytes, and finally smooth muscle cell migration and proliferation within the resolving thrombus [5–7]. The initiation, propagation and

Abbreviations: ADP, adenosine diphosphate; ANOVA, analysis of variance; AUC, area under the curve; PAI-1, plasminogen activator inhibitor type 1; PCI, percutaneous coronary intervention; t-PA, tissue-type plasminogen activator.

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stabilization of acute stent thrombosis and in-stent restenosis are therefore dependent on several components: platelet aggregation, endothelial function, coagulation and fibrinolysis.

The purpose of the present study was to identify potential factors that may be implicated in the predisposition to acute stent thrombosis or in-stent restenosis using a case-control methodology. We assessed endothelium-dependent vasomotion, platelet aggregation and the acute endogenous fibrinolytic capacity in patients who have developed thrombotic complications following percutaneous coronary intervention (PCI) and stent implantation.

1. Materials and methods

1.1. Subjects

Twenty-six patients who had undergone PCI with stent implantation at least 6 months previously and 10 age- and sex-matched healthy control subjects were recruited into the study. In the patient group, 16 had developed complications of acute stent thrombosis within 48 h of stent implantation ($n=6$) or in-stent restenosis within 6 months of intervention ($n=10$), and 10 had no clinical evidence of stent thrombosis or restenosis at least 1 year following the procedure. Patients with procedural complications or suboptimal stent insertion were excluded. All patients received thienopyridine therapy for 4 weeks after undergoing PCI and none received a glycoprotein IIb/IIIa receptor antagonist during the procedure.

All subjects abstained from alcohol for 24 h, and from food and caffeine-containing drinks for at least 4 h before the study, and medications were withheld on the day of the study. All studies were performed in a quiet, temperature-controlled room maintained at 22–25 °C. The investigation was performed with the approval of the local ethics committee and conformed with the principles outlined in the Declaration of Helsinki, and with the written informed consent of each subject.

1.2. Drugs administration

Pharmaceutical-grade substance P (Clinalfa, Läufelfingen, Switzerland), acetylcholine (Cibavision Ophthalmics, Southampton, UK) and sodium nitroprusside (David Bull Laboratories, Faulding, UK) were administered following dissolution in saline (0.9%: Baxter Healthcare). All solutions were freshly prepared on the day of study.

1.3. Hemodynamic measurements

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges as previously described [8,9]. Blood pressure was monitored in the non-infused arm at intervals throughout each study with a semiautomated noninvasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical).

1.4. Fibrinolytic assays

Venous blood (10 ml) was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool® Stabilyte™, Umeå, Sweden; for t-PA assays) and trisodium citrate (Monovette®, Sarstedt, Nümbrecht, Germany; for PAI-1 assays) tubes and centrifuged at $2000 \times g$ for 30 min at 4 °C. Platelet-free plasma was decanted and stored at −80 °C before assay. Plasma tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) antigens were determined as previously described [8] using enzyme-linked immunosorbent assays (Coaliza t-PA and Coaliza PAI-1, Chromogenix, Mölndal, Sweden) and plasma t-PA activity using a photometric method (Coatest t-PA, Chomogenix).

1.5. Platelet aggregometry and thrombophilia screen

Fasting venous blood (30 ml) was collected into trisodium citrate tubes (Monovette®) and immediately centrifuged at $120 \times g$ for 10 min to obtain platelet-rich plasma, which was aspirated, adjusted to give a platelet count of 250×10^9/l and pre-warmed to 37 °C. Aggregation studies were performed

Table 1
Baseline characteristics

	Acute stent thrombosis ($n=6$)	In-stent restenosis ($n=10$)	Control patients ($n=10$)	Healthy subjects ($n=10$)
Sex (male/female)	2:4	9:1	8:2	9:1
Age	65 ± 3	62 ± 2	63 ± 2	58 ± 3
Current smoker, % (*n*)	17 (1)	20 (2)	20 (2)	20 (2)
Diabetes mellitus, % (*n*)	17 (1)	30 (3)	20 (2)	0 (0)
Hypertension, % (*n*)	50 (3)	60 (6)	30 (3)	0 (0)
Three-vessel disease, % (*n*)	33 (2)	30 (3)	20 (2)	0 (0)
Normal or mild left ventricular impairment, % (*n*)	84 (5)	90 (9)	80 (8)	100 (10)
Average stent diameter, mm	3.3 ± 0.2	3.0 ± 0.1	3.2 ± 0.2	
Average stent length, mm	16 ± 2	18 ± 2	20 ± 2	
Medical therapy				
Aspirin, % (*n*)	100 (6)	100 (10)	100 (10)	0 (0)
β-adrenoceptor antagonists, % (*n*)	50 (3)	50 (5)	60 (6)	0 (0)
Angiotensin-converting enzyme inhibitors, % (*n*)	33 (2)	60 (6)	40 (4)	0 (0)
Statins, % (*n*)	100 (6)	80 (8)	80 (8)	0 (0)
Calcium channel blockers, % (*n*)	50 (3)	60 (6)	10 (1)	0 (0)
Oral nitrates, % (*n*)	50 (3)	30 (3)	20 (2)	0 (0)

Mean ± S.E.M.

Table 2
Infused forearm blood flow during substance P, acetylcholine and sodium nitroprusside infusion

Drug infusion	Infused forearm blood flow (ml/100 ml/min)			
	Acute stent thrombosis ($n=6$)	In-stent restenosis ($n=10$)	Control patients ($n=10$)	Healthy subjects ($n=10$)
Baseline	1.7 ± 0.4	2.5 ± 0.3	2.4 ± 0.2	2.8 ± 0.5
Substance P (pmol/min)				
4	8.6 ± 0.5	9.6 ± 1.1	9.7 ± 1.2	7.9 ± 1.0
8	9.7 ± 0.7	10.6 ± 1.1	11.0 ± 1.1	9.7 ± 1.3
16	11.6 ± 1.3*	11.3 ± 1.4*	13.2 ± 1.6*	12.4 ± 1.9*
Acetylcholine (μg/min)				
5	7.7 ± 1.8	5.7 ± 1.2	6.2 ± 0.9	6.2 ± 1.3
10	9.7 ± 2.3	6.3 ± 1.4	6.9 ± 1.0	7.3 ± 1.1
20	14.4 ± 3.1‡	9.2 ± 2.0‡	9.0 ± 1.5†	9.4 ± 2.1‡
Sodium nitroprusside (μg/min)				
2	9.7 ± 2.9	7.3 ± 1.0	7.3 ± 0.6	8.4 ± 0.9
4	11.6 ± 2.7	11.1 ± 1.2	10.1 ± 0.9	10.9 ± 1.3
8	13.3 ± 2.6‡	13.4 ± 1.4*	12.6 ± 1.4*	14.4 ± 2.1*

ANOVA for dose response.
* $P<0.0001$.
† $P<0.001$.
‡ $P<0.05$.

on the platelet-rich plasma, 30–40 min after blood sampling, using a standard optical technique (Chronolog Ca560 aggregometer; Labmedics, Stockport, UK) as described previously [10], using the following agonists: adenosine diphosphate (ADP, 0.5–10 μmol/l), the thromboxane A2 analogue U46619 (0.5–6 μmol/l), thrombin (200–1000 mU/l) and collagen (1–5 μg/ml). Responses were for 5 min after addition of agonists, and the peak response recorded and adjusted for platelet count (250×110^9/l). Platelet count and hematocrit were measured using an automated Coulter counter (Act. 8 Coulter Counter; Beckman-Coulter, High Wycombe, UK).

Venous blood (12 ml) were collected in potassium EDTA and trisodium citrate (Monovette®) tubes and screened for anti-thrombin, protein S and protein C deficiencies, factor V (Leiden) and prothrombin A20210G genotypes and anti-phospholipid antibodies.

1.6. Study design

Subjects attended following a 4-h fast and then rested recumbent throughout the study. Strain gauges and cuffs were applied, and 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% lidocaine (Xylocaine; Astra Pharmaceuticals) local anesthesia and attached to a 16-gauge epidural catheter (Portex). Patency was maintained by infusion of saline via a MS2000 syringe infusion pump (Graesby Medical, Watford, UK). Venous cannulae (17-gauge) were inserted into large

Table 3
Plasma t-PA and PAI-1 concentrations and release during substance P infusion

	Substance P (pmol/min)	Acute stent thrombosis ($n=6$)		In-stent restenosis ($n=10$)		Control patients ($n=10$)		Healthy subjects ($n=10$)	
		Infused arm	Non-infused arm	Infused arm	Non-infused arm	Infused arm	Non-infused arm	Infused arm	Non-infused arm
t-PA antigen (ng/ml)	Baseline	4.9 ± 0.9	4.8 ± 0.9	4.3 ± 0.7	4.1 ± 0.6	4.7 ± 0.3	4.9 ± 0.5	5.1 ± 0.4	5.4 ± 0.5
	4	7.2 ± 1.1	5.2 ± 1.2	5.4 ± 0.8	4.2 ± 0.6	5.8 ± 0.5	4.7 ± 0.4	6.1 ± 0.6	5.4 ± 0.5
	8	7.9 ± 1.1	4.9 ± 1.2	6.0 ± 0.7	4.7 ± 0.6	6.5 ± 0.6	4.7 ± 0.4	7.0 ± 0.8	5.5 ± 0.5
	16	10.3 ± 0.8*	5.6 ± 1.3	8.8 ± 1.5*	4.7 ± 0.6	7.6 ± 0.8†	4.9 ± 0.4	8.5 ± 1.3	7.6 ± 1.5
Estimated t-PA antigen release (ng/100 ml/min)	Baseline	0.1 ± 0.5		0.2 ± 0.4		− 0.4 ± 0.4		− 0.5 ± 0.4	
	4	11.2 ± 4.1		6.8 ± 2.5		6.9 ± 2.8		3.1 ± 1.3	
	8	18.7 ± 7.7		6.9 ± 2.5		12.2 ± 3.3		7.8 ± 3.3	
	16	34.4 ± 10.1*		25.3 ± 7.5†		19.6 ± 3.3‡		5.9 ± 2.1*	
Net t-PA antigen release (AUC)		47.1 ± 13.9		26.4 ± 6.6		28.6 ± 8.0		13.6 ± 4.1	
t-PA activity (IU/ml)	Baseline	2.1 ± 0.5	1.8 ± 0.4	2.0 ± 0.3	2.1 ± 0.5	1.2 ± 0.2	1.2 ± 0.2	0.8 ± 0.2	1.0 ± 0.2
	4	5.3 ± 1.0	2.2 ± 0.4	4.6 ± 0.8	2.5 ± 0.6	2.4 ± 0.5	1.29 ± 0.2	2.3 ± 0.5	1.0 ± 0.2
	8	6.7 ± 1.1	2.1 ± 0.4	4.7 ± 0.9	2.7 ± 0.6	3.0 ± 0.6	1.88 ± 0.3	3.8 ± 1.2	1.4 ± 0.3
	16	7.6 ± 1.1†	3.8 ± 0.8	7.0 ± 0.9‡	3.6 ± 0.9	5.5 ± 1.0†	2.04 ± 0.4	5.9 ± 1.7	1.7 ± 0.4
Estimated t-PA activity release (IU/100 ml/min)	Baseline	0.1 ± 0.2		− 0.1 ± 0.4		0.0 ± 0.1		− 0.3 ± 0.1	
	4	16.8 ± 4.6		11.1 ± 3.6		6.6 ± 2.8		5.6 ± 1.8	
	8	27.9 ± 5.9		11.3 ± 3.8		6.1 ± 2.3		16.1 ± 8.5	
	16	25.5 ± 5.5†		18.3 ± 4.2†		25.8 ± 5.3‡		31.5 ± 13.6*	
Net t-PA activity release (AUC)		57.5 ± 10.9		31.5 ± 8.2		25.6 ± 7.7		37.0 ± 16.7	
PAI-1 antigen (ng/ml)	Baseline	33 ± 4	36 ± 4	28 ± 7	30 ± 7	43 ± 8	43 ± 7	36 ± 5	36 ± 5
	16	28 ± 2	33 ± 4	32 ± 7	33 ± 7	42 ± 7	46 ± 8	36 ± 5	34 ± 5

Mean ± S.E.M. ANOVA for dose response.
* $P<0.05$.
† $P<0.01$.
‡ $P<0.001$.

subcutaneous veins of the antecubital fossae of both arms. After 30 min equilibration with saline infusion, intra-arterial substance P was administered at 4, 8, 16 pmol/min, acetylcholine at 5, 10, 20 μg/min and sodium nitroprusside at 2, 4, 8 μg/min for 10 min at each dose [8,11]. The drugs were separated by 20 min of saline infusion and administered in a randomized order. All infusions were given at a constant infusion rate of 1 ml/min. Venous samples were taken at baseline and during infusion of each substance P dose but not during sodium nitroprusside or acetylcholine infusion since they do not affect plasma t-PA or PAI-1 concentrations in this forearm model [8,10,12].

1.7. Data analysis and statistics

Plethysmographic and aggregometry data were extracted from the Chart data files and forearm blood flow was calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel 97, Microsoft). Estimated net release of t-PA antigen and activity was defined previously [8] as the product of the infused forearm plasma flow (based on the mean hematocrit and the infused forearm blood flow) and the concentration difference between the infused and noninfused arms. Data was examined, where appropriate, by analysis of variance (ANOVA) with repeated measures and two-tailed paired Student's *t*-test using Statview (SAS Institute). All results are expressed as mean ± S.E.M. Statistical significance was taken at the 5% level.

2. Results

The baseline characteristics of all subjects are shown in Table 1. The patient groups are matched for age, smoking history, diabetes mellitus, hypertension and severity of coronary artery disease although there appeared to be a greater proportion of female subjects in the acute stent thrombosis group. No significant abnormalities were demonstrated in the thrombophilia screen in all study subjects.

2.1. Endothelium-dependent vasomotion

There were no significant changes in heart rate, blood pressure and non-infused forearm blood flow during drug infusion in all studies. Forearm blood flow increased in a dose-dependent manner during substance P, acetylcholine and sodium nitroprusside infusions ($P<0.05$, ANOVA) (Table 2). However, there were no significant differences between the four groups.

2.2. Endogenous fibrinolysis

Compared to the non-infused arm, substance P caused dose-dependent increases in plasma t-PA antigen and activity concentrations in the infused arm in all subjects ($P<0.05$, Table 3), but this increase was not different between the groups. Patients with acute stent thrombosis had an apparently higher t-PA antigen release but this did not achieve statistical significance nor was this seen with t-PA activity. Post-hoc analysis demonstrated a significant reduction of t-PA antigen and activity release in current smokers compared to non-smokers ($P<0.05$, Fig. 1).

2.3. Platelet aggregation

All patients were on aspirin therapy and had reduced platelet aggregation in response to collagen ($P<0.01$) compared to healthy volunteers (Fig. 2). There was no significant difference in platelet aggregation to thrombin, collagen or U46619 between the patient groups (Fig. 2).

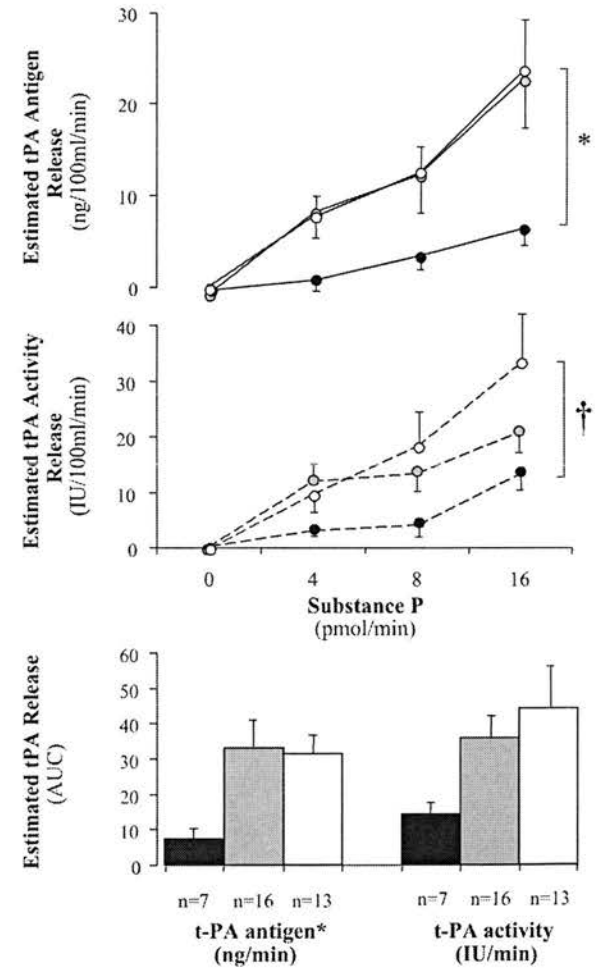

Fig. 1. Estimated net release of t-PA antigen and activity in current smokers (black), ex-smokers (grey) and non-smokers (white). Lower panel represents area under the curve (AUC) for the response. ANOVA for smoking status *$P<0.01$, †$P<0.05$.

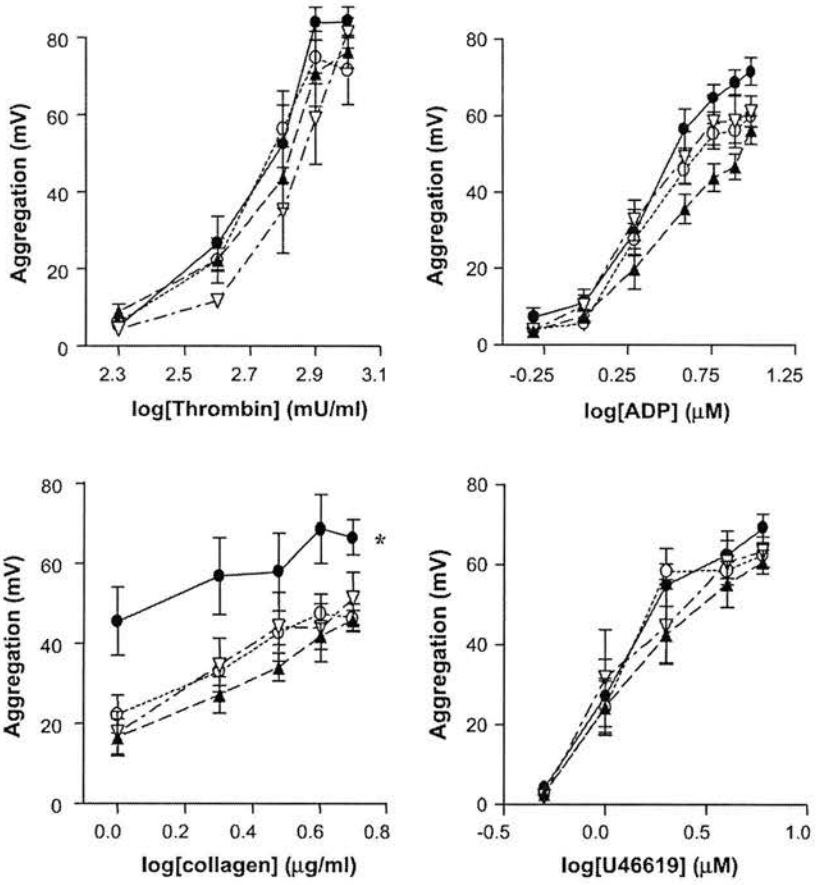

Fig. 2. Platelet aggregation in response to thrombin, collagen, ADP and U46619 in patients with acute stent thrombosis (open inverted triangles), in-stent restenosis (closed triangles) and uncomplicated PCI with stent implantation (open circles), and healthy matched controls (closed circles). $P<0.001$ for each dose response. *$P<0.01$ controls vs. patients.

However, platelet aggregation was increased in patients with acute stent thrombosis in response to ADP compared to in-stent restenosis ($P<0.001$), although this was not significant when compared to the control group ($P=0.19$).

3. Discussion

We have assessed three critical aspects of vascular function in patients who have undergone PCI. We have found no evidence to indicate that endothelial vasomotor or fibrinolytic function plays a major role in the pathogenesis of acute stent thrombosis or in-stent restenosis. Moreover, in vitro platelet function appeared to be normal with little evidence of alterations in platelet sensitivity to a number of agonists. In contrast, we have been able to confirm our previous findings [9,13] of the marked inhibition of acute t-PA release in current smokers. We conclude that endothelial and platelet function do not appear to be major determinants of acute stent thrombosis or in-stent restenosis.

3.1. Endothelial vasomotor function

The endothelium plays a critical role in the regulation of vasomotor tone [14,15] and its injury or dysfunction is an important contributing factor in atherothrombosis. There is progressive impairment of endothelium-dependent vasodilatation with the development of coronary atherosclerosis [16] and its associated risk factors, such as hypercholesterolemia [17], smoking [9] and diabetes mellitus [18]. In this study, we assessed forearm blood flow following intra-arterial infusions of the endothelium-dependent vasodilators, substance P and acetylcholine, and the endothelium-independent vasodilator, sodium nitroprusside. We found no differences in the forearm blood flow responses either between the patient groups or comparing the patient and healthy control groups. Given the absence of risk factors and clinically evident disease, one would have anticipated greater endothelium-dependent vasodilatation in the healthy control subjects. However, ageing has a marked effect on the regulation of basal [19] and stimulated [20] endothelium-dependent vascular tone and we studied a predominantly elderly population with a mean age of 62 years. Moreover,

we recognise that subjects who are apparently healthy in this age group commonly have subclinical atherosclerosis that cannot be excluded in the absence of invasive investigation such as coronary angiography.

3.2. Endogenous fibrinolysis

The regulated release of endothelial t-PA is an important mechanism in the defence against intravascular thrombosis especially in the coronary circulation [21]. Several investigators have suggested that impaired endogenous fibrinolysis is associated with restenosis after PCI [22,23], and in particular, a rise in plasma PAI-1 antigen concentrations [24]. In the present study, we were unable to find any evidence of impaired endogenous fibrinolysis in our patients with no apparent differences in plasma PAI-1 antigen concentrations or acute endothelial t-PA release. However, when stratifying the subjects according to smoking habit, post-hoc analysis indicated that acute t-PA release was markedly impaired in smokers compared to non-smokers. This confirms our previous findings of impaired t-PA release in smokers [9,13] and gives support to our conclusions that, rather than a lack of power, there appears to be no major impairment of endogenous fibrinolysis in patients with acute stent thrombosis or in-stent restenosis.

3.3. Platelet function

Platelet adherence to the arterial wall occurs within minutes of arterial injury. Pathological studies [25] in both porcine coronary arteries and in human saphenous vein grafts have shown that the earliest vascular response to stent implantation is extensive platelet deposition. However, despite important reductions in periprocedural events [26,27], the glycoprotein IIb/IIIa receptor antagonist abciximab does not reduce in-stent restenosis [28]. Consistent with this observation, platelet aggregation in response to thrombin, ADP, the thromboxane A2 analogue U46619 or collagen was unaffected in our study patient populations. In keeping with long-term aspirin use, patients in all three groups demonstrated reduced collagen-dependent platelet aggregation in comparison with healthy subjects. Again, this suggests that, rather than a lack of power, there appears to be no major difference in platelet aggregation in patients with acute stent thrombosis or in-stent restenosis. Although assessment of platelet function should ideally be performed after aspirin has been withheld for at least 1 week, aspirin use was unavoidable in our patient population.

3.4. Study limitations

There are several potential limitations to our clinical study. First, complications from stent implantation may arise due to procedural difficulties or suboptimal stent deployment. However, we were careful to exclude such patients with overt technical problems during the PCI procedure. Second, acute stent thrombosis is a fortunately rare complication of PCI but this makes recruitment of such patients problematic. This is reflected in the modest number of patients with acute stent thrombosis in our study and means that we lack sufficient power to address the influence of all the individual variables associated with thrombosis, particularly given the higher proportion of female patients in this group. The relatively low number of patients and subjects in the whole study may also raise the potential possibility of a type II error. Third, the assessment in our study is performed at least 6 months following PCI and stent implantation in order to exclude the development of late restenosis in the control patient group. Whilst this avoids the influence of acute confounding factors, we may have missed a transient impairment in endothelial or platelet function. It is therefore possible that changes in platelet behaviour, fibrinolysis or vascular function may occur in the acute phase of stent-thrombosis and this study does not definitely exclude that changes in these factors contribute to the development of acute stent thrombosis. Finally, we used a clinical diagnosis of restenosis and cannot exclude a degree of subclinical angiographic restenosis in the control patient group.

In conclusion, our study suggests that endothelial dysfunction, platelet aggregation and endogenous fibrinolysis do not appear to play a major role in the pathogenesis of acute stent thrombosis or in-stent restenosis.

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