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Impairment of Endothelial Thromboprotective Function by Haemodynamic and Inflammatory Stress

Implications for hypertensive disease

Erik Ulfhammer

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To my family

Impairment of Endothelial Thromboprotective Function by Haemodynamic and Inflammatory Stress

Implications for hypertensive disease

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ABSTRACT

The physiologically most important activator of intravascular fibrinolysis is tissue-type plasminogen activator (t-PA). The endothelium synthesizes and stores t-PA and regulated release of the enzyme is an important local protective response to prevent thrombus extension. Previous work by our group has shown that both patients with primary and secondary hypertension have a reduced capacity to release t-PA upon stimulation, a defect that is likely to contribute to the enhanced risk for arterial occlusion and tissue infarction in these subjects. The mechanism of this impairment is unclear although our experimental studies have indicated that it could be a direct effect of the elevated blood pressure.

In order to investigate if the impairment could be reversed by lowering the blood pressure, we used the perfused-forearm model to examine hypertensive subjects for stimulated t-PA release before and after antihypertensive treatment. The findings show that the capacity for stimulated t-PA release can be significantly improved by blood pressure lowering. Treatment increased the amount of t-PA released and also improved the rapidity of the response. The changes were of similar magnitude regardless of treatment with the angiotensin converting enzyme inhibitor lisinopril or the calcium antagonist felodipine, suggesting that the improvement was related to the blood pressure effect *per se*.

To examine the underlying mechanism of blood pressure-induced suppression of t-PA, we explored the potential involvement of the two main haemodynamic forces tensile stress and shear stress. Using *in vitro* biomechanical experimental models and cultured endothelial cells we observed suppressed t-PA gene expression and protein secretion in response to prolonged cyclic strain stimulation and a magnitude dependent suppression of t-PA transcript with prolonged laminar shear stress. Moreover, all reductions of t-PA were consistently followed by inductions of the main inhibitor of t-PA, plasminogen activator inhibitor type 1 (PAI-1).

Further, as hypertension is often associated with a low-grade inflammation, we investigated the impact of the prototypic proinflammatory cytokine tumor necrosis factor-α (TNF-α) on t-PA expression. Prolonged stimulation of cultured endothelial cells was observed to suppress t-PA gene and protein expression. Mechanistic experiments with pharmacologic inhibitors showed that the inhibitory effect was nuclear factor-κB (NF-κB) and p38 mitogen-activated protein kinase (p38 MAPK) dependent and indicated that potential effector molecules might be the transcription factors NF-κB and CREB interacting with the t-PA κB and CRE promoter elements, respectively.

In conclusion, these findings show that the impaired capacity to release t-PA in hypertensive subjects is directly related to the elevated blood pressure. Data from experimental studies indicate that this impaired fibrinolytic response could be an effect of an enhanced tensile, shear and inflammatory stress acting on the endothelium.

Key words: tissue-type plasminogen activator, endothelium, fibrinolysis, hypertension, shear stress, strain, antihypertensive agents, inflammation, TNF-α, NF-κB, MAPK

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LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, identified in the text by their Roman numerals:

I Ridderstråle W, Ulfhammer E, Jern S, Hrafnkelsdóttir T. Impaired capacity for stimulated fibrinolysis in essential hypertension is restored by antihypertensive therapy.
Hypertension 2006;47:686-91.

II Ulfhammer, E, Ridderstråle W, Andersson M, Karlsson L, Hrafnkelsdóttir T, Jern S. Prolonged cyclic strain impairs the fibrinolytic system in cultured vascular endothelial cells.
Journal of Hypertension 2005;23:1551-7.

III Ulfhammer E, Carlström M, Bergh N, Larsson P, Karlsson L, Jern S. Steady laminar shear stress suppresses tissue-type plasminogen activator expression in vascular endothelial cells.
Submitted for publication.

IV Ulfhammer E, Larsson P, Karlsson L, Hrafnkelsdóttir T, Bokarewa M, Tarkowski A, Jern S. TNF-alpha mediated suppression of tissue type plasminogen activator expression in vascular endotheial cells is NF-kappaB- and p38 MAPK-dependent.
Journal of Thrombosis and Haemostasis 2006;4:1781-9.

CONTENTS

ABBREVIATIONS

ACE	angiotensin converting enzyme
AP-1	activator protein-1
ANOVA	analysis of variance
ATF	activating transcription factor
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CRE	cAMP response element
CREB	CRE-binding protein
C_T	threshold cycle
CTF	CCAAT-binding transcription factor
DNA	deoxyribonucleic acid
EBM-2	endothelial basal medium-2
EGM-2	endothelial growth medium-2
Egr-1	early growth response-1
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
ET-1	endothelin-1
FBF	forearm blood flow
FBS	fetal bovine serum
FPF	forarm plasma flow
FVR	forearm vascular resistance
GAPDH	glyceraldehyd 3-phosphate dehydrogenase
HAEC	human aortic endothelial cells
HUVEC	human umbilical vein endothelial cells
ICAM-1	intracellular adhesion molecule-1
IL-1β	interleukin-1β
JNK	c-jun N-terminal kinase
kD	kilo Dalton
MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MEKK	MEK kinase
mRNA	messenger RNA
NF1	nuclear factor 1
NF-κB	nuclear factor-κB
NO	nitric oxide
PAF	platelet activating factor
PAI-1	plasminogen activator inhibitor-1
PGI_2	prostacyclin
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SEM	standard error of the mean
Sp1	specificity protein 1
SSRE	shear stress responsive element
TFPI	tissue-factor pathway inhibitor
TNF-α	tumor necrosis factor-α
t-PA	tissue-type plasminogen activator
TRE	PMA responsive element
tRNA	transfer RNA
u-PA	urokinase-type plasminogen activator
VCAM-1	vascular cell adhesion molecule-1

INTRODUCTION

Cardiovascular diseases, such as myocardial infarction and ischemic stroke, are currently the leading cause of death and illness in developed countries, and acute vascular syndromes are becoming a major concern worldwide [1]. Myocardial infarctions and a substantial part of ischemic strokes are caused by activation of intravascular clotting mechanisms that, when unopposed, rapidly can progress into the formation of an occluding arterial thrombus. To protect against this, the vascular endothelium of healthy individuals has the capacity to activate an acute fibrinolytic response if intravascular clotting should occur. *In vivo* studies from our group have shown that the major determinant of the local fibrinolytic response is the capacity of the endothelium to release the key fibrinolytic enzyme tissue-type plasminogen activator (t-PA) [2]. This indicates that in case of an imbalanced endogenous fibrinolysis, due to a reduced capacity for t-PA release, intravascular thrombus formation may propagate, ultimately leading to arterial occlusion and tissue infarction. In experimental studies, our group has previously observed a suppressed endothelial expression of t-PA when the blood vessel wall was exposed to increased intraluminal pressure [3]. It was hypothesized that this could be a mechanism for the increased incidence of atherothrombosis in human hypertension, a hypothesis that was supported by studies showing that both patients with essential hypertension [4] and chronic renal failure with hypertension [5] had an impaired capacity for acute t-PA release upon stimulation. Against this background, this thesis focuses on the underlying mechanisms of the impaired t-PA response in hypertensive subjects, with attention being paid to the influence of haemodynamic and inflammatory stress.

The vascular endothelium

The luminal side of all blood vessels is lined with a multifunctional monolayer of cells, the vascular endothelium, which may be regarded as an organ dispersed over the entire body [6]. The total surface area of the endothelium has been reported to vary between 350 and 1000 m^2 and with a weight of 0.1 and 1.5 kg [6-8]. The endothelium occupies a strategic position between the nutritive blood flow and the metabolically demanding tissue. Also, the endothelium senses mechanical, chemical, and humoral stimuli, and responds by synthesis and release of a wide range of biologically active mediators. Thus, it regulates vascular tone by release of vasoactive substances such as nitric oxide (NO), prostacyclin (PGI_2), and endothelin-1 (ET-1), some of which also possess antithrombotic properties (NO, PGI_2). Furthermore, the endothelium has a pivotal role in maintenance of blood fluidity by expressing antithrombotic and fibrinolytic properties. Surface-expressed compounds like tissue-factor pathway inhibitor (TFPI), thrombomodulin, heparan sulfate, ecto-ADPase, and protein S have antithrombotic and anticoagulating properties. The fibrinolytic functions include the release of t-PA, enabling initiation of fibrinolysis, while endothelial receptors for plasminogen and plasminogen activators further enhance that process. Stimulation of the endothelium by products involved in the intravascular clotting process induces a regulated secretion of t-PA [9, 10] and TFPI [11], together with release of vasorelaxing and anti-aggregatory mediators such as NO [12] and vasoactive eicosanoids [13].

The fibrinolytic system

The endogenous fibrinolytic system protects the circulation from intravascular fibrin formation and thrombosis, thereby acting as a counter-regulatory mechanism to the coagulation cascade. This is illustrated by the observation that in about 30% of events with acute myocardial or cerebral infarction the infarct-related artery spontanously reperfuses [14, 15]. The fibrinolytic system is regulated by circulating factors and factors released from the vascular endothelium. Fibrin is degraded by the protease plasmin, which in turn requires plasminogen activators for conversion from the proenzyme plasminogen [16]. There are two immunological distinct plasminogen activators; t-PA and urokinase-type plasminogen activator (u-PA) [16-18]. In the vascular compartment, t-PA induced activation of plasminogen is the physiologically most important trigger of fibrinolysis [17, 19-21]. u-PA appears mainly to be involved in later stages of fibrin dissolution and processes involving cell movement and tissue remodeling [21, 22]. The activity of the fibrinolytic enzymes is regulated by serine protease inhibitors (serpins). The main inhibitor of plasmin is α_2-antiplasmin, while plasminogen activator inhibitor type-1 (PAI-1) is considered to be the physiologically most important inhibitor of t-PA and u-PA in plasma [23-25]. Other circulating inhibitors of plasminogen activators, such as C1-inhibitor, α_2-macroglobulin, and α_1-antitrypsin are probably of less importance under physiological conditions [26-28].

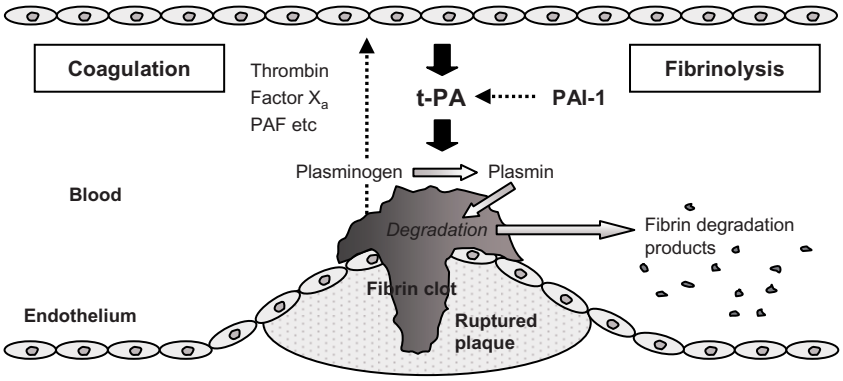

Figure 1. The intravascular fibrinolytic system. t-PA converts the proenzyme plasminogen to plasmin, which in turn degrades the fibrin structure of a forming clot, thereby aiding its dissolution. As substances formed during the clotting process are potent triggers of t-PA release, this works as an important counter-regulatory mechanism to prevent the formation of intraluminal thrombi and tissue ischemia. PAI-1 is the main inhibitor of t-PA in the vascular compartment.

t-PA

The central role of t-PA in fibrinolysis

The crucial process in initiating an endogenous fibrinolytic response is the release of t-PA from the vascular endothelium. In contrast to most other serine proteases, t-PA is released as an active enzyme. The proteolytic activity of t-PA is greatly enhanced

by fibrin [29, 30], and t-PA associated to fibrin is protected from complex formation with inhibitors. In addition, t-PA already present during thrombus formation is much more potent in inducing thrombus dissolution than when added later, *i.e*. to a manifest thrombus [19, 20]. Thus, endogenous local release of t-PA is likely to be an important defense mechanism against thrombosis and tissue ischemia.

The importance of t-PA release has been confirmed in studies on t-PA deficient mice [31-33]. When thrombus formation was induced in the carotid artery by endothelial injury, the rate of persistent occlusion was increased in t-PA deficient as compared to wild-type mice [33]. In line with this, gene therapy that induced local overexpression of t-PA, without systemic effects, prevented arterial thrombosis in an *in vivo* rabbit model [34]. These observations also extend to the clinical setting in which recombinant t-PA is used in the treatment of myocardial infarction and ischemic stroke, a therapy that results in clot dissolution and reduced mortality. Recently, the clinical importance of a functional t-PA response was further confirmed, as subjects with a low capacity for t-PA release, due to a polymorphism in t-PA gene, were found to have a more than 3-fold adjusted increased risk for myocardial infarction [35].

Synthesis and secretion of t-PA

Endothelial cells synthesize and store t-PA, and are considered to be the main source of circulating t-PA [36]. The serine protease enters the circulation in an active single-chain form and the release follows both a constitutive and a regulated pathway [37, 38]. In constitutive secretion, the newly synthesized protein continously leaves the Golgi apparatus in transport vesicles to fuse with the cell membrane. During regulated release, large amounts of t-PA are released from an intracellular storage pool. *In vivo* and *in vitro* data show that the amount of t-PA released by both these pathways is proportional to t-PA synthesis [10]. Synthesis of t-PA in cultured human endothelial cells is enhanced by activators of protein kinase C (PKC; phorbol esters, thrombin, and histamin), cAMP elevating agents in combination with ativators of PKC (*e.g.* forskolin), retinoids, short-chain fatty acids *(e.g.* sodium butyrate), and triazolobenzodiazepines [39]. A decrease in t-PA synthesis has been observed with reactive oxygen species [40], while the reported effects of cytokines and haemodynamic stress have been somewhat conflicting.

Regulated release of t-PA

A key step in the endogenous fibrinolytic system is the immediate stimulated release of active t-PA from specialized intracellular storage granules [9, 41]. This storage pool enables release upon stimulation and a several-fold increase in secretion rate if needed *e.g*. during thrombus formation. Many studies have focused on trying to elucidate the mechanisms involved in triggering this release. However, both *in vitro* and *in vivo* studies addressing this issue have proven to be difficult. *In vitro*, endothelial cells contain only small storage pools of t-PA [42] which, along with the unfavorable surface-volume ratios that are generated *in vitro*, means that specially adapted techniques are required to detect the very low t-PA concentrations (picograms) produced by acute release from endothelial cells in culture. The use of *in vivo* models for studies of regulated t-PA release is therefore preferable, but appropriate human experimental

approaches have been lacking. Therefore, in 1994 our group established a regional *in vivo* model, the perfused-forearm model, which allows direct measurement of local endothelial t-PA release rates without the confunding effect of liver clearance or inhibition [43, 44]. Regulated release of t-PA can be induced by a number of substances with the common denominator that they activate endothelial G-protein coupled cell surface receptors resulting in enhanced cytoplasmic Ca^{2+} [45]. Most importantly, several products formed during the process of thrombus formation, *i.e*. thrombin, bradykinin, factor X_a, and platelet activating factor (PAF) are potent activators of this response [9, 10, 46]. The same holds true for metabolites of tissue ischemia [47], mental stress [44] and sympathoadrenal activation [48]. Regulated release of t-PA can also be induced by a variety of endogenous and exogenous endothelial receptor agonists, including *e.g.* norephinephrine [43], methacoline [43], substance P [49], bradykinin [50], and desmopressine [51].

Plasma levels of t-PA

Prospective studies consistently show that an elevated systemic plasma level of t-PA antigen predicts both myocardial infarction and ischemic stroke [52-55]. This may seem paradoxical, given the thromboprotective role of t-PA. However, as stated before, it is the capacity for regulated t-PA release that is the crucial mechanism in determining the endogenous fibrinolytic response [2]. Measurements of plasma t-PA antigen reflects the sum of the different molecular forms of t-PA, *i.e*. t-PA in complex with inhibitors, mainly PAI-1, and uncomplexed, active t-PA [56-59]. In plasma, the complex-bound and thereby biologically inactive form of t-PA represents the vast majority of total t-PA antigen, whereas only approximately 20% circulates in its free and active form [56-58]. Furthermore, the half-life of t-PA in plasma is short, only 3-5 min [60], and it is cleared from the circulation by receptor-dependent mechanisms in the liver [61]. The clearance of t-PA is dependent on liver blood flow and on how much of it is complex-bound to PAI-1, since active t-PA is cleared more rapidly than the t-PA that is bound to PAI-1 [60]. It follows that an increased plasma concentration of PAI-1 will be paralleled by an increased plasma concentration of t-PA antigen despite an unchanged endothelial secretion rate, thus indicating a reduced rather than enhanced local fibrinolytic capacity. In line with this, plasma t-PA antigen shows a negative correlation to plasma t-PA activity [62]. Thus, the systemic plasma level of t-PA antigen does not reflect the endothelial secretion rate of the protein.

Regulation of t-PA gene expression

The human gene coding for t-PA is localized on chromosome 8 [63, 64]. It consists of 14 exons coding for 527-530 amino acids of the mature 65-75 kD protein [36, 65, 66]. Several studies have shown that t-PA synthesis is principally regulated at the level of transcription [39, 67]. The transcription is mainly mediated by a TATA less promoter [68], although earlier studies reported on a TATA dependent initiation site 110 bp upstream of this site [65, 66]. However, in all cell types tested, the TATA independent initiation site is the predominant one, with an approximately ten-fold higher transcription rate in endothelial cells as compared to the TATA dependent counterpart [68, 69]. Positions in the t-PA gene in this thesis are therefore numbered relative to the TATA less site.

Several *cis*-acting elements have been identified both within the proximal t-PA promoter and at far upstream locations, thereby highlighting the complex nature of the transcriptional regulation of the t-PA gene. TATA less promoters depend on the transcription factor specificity protein 1 (Sp1) for the recruitment of the transcription initiation complex. In line with this, several potential Sp1 binding motifs have been identified in the proximal t-PA promoter (GC box I, II, III). Of these, GC box II (bp -71 to -65) and III (bp -48 to -42) have been shown to bind Sp1 [69, 70], and a strict correlation between the binding of nuclear proteins to GC box III and t-PA expression has been reported for several cell types. In addition to GC box III, a cyclic adenosine monophosphate (cAMP) response element (CRE)-like site (bp -223 to -216) has been shown to be essential for both basal and inducible transactivation of the t-PA promoter in endothelial cells [69]. This DNA element, also referred to as a phorbol 12-myristate 13-acetate (PMA) responsive element (TRE) [70], provides a binding site for transcription factors belonging to the activator protein-1 (AP-1) and CRE binding protein (CREB)/activating transcription factor (ATF) families, but cell type specific binding variations for the individual components have been described [69-71]. Moreover, a consensus site for the family of CCAAT-binding transcription factors (CTF; also denoted nuclear factor 1, NF1) has been identified (bp -202 to -188) [70].

Besides the described elements of the proximal t-PA promoter, *cis*-acting elements located further upstream have been characterized. These include a functional κB element, which was recently found in the t-PA gene of human neuronal cells (bp -3081 to -3072) [72], and a potential shear stress responsive element (SSRE; bp -1060 to -1055) [73]. The t-PA gene is also under control of a well-characterized multihormone responsive enhancer located 7.1 to 8.0 kb upstream of the transcription start site [74].

PAI-1

PAI-1 is a 50 kD serpin, consisting of 379 aminoacids. PAI-1 is synthesized and secreted as an active inhibitor, but is spontanously converted into a latent, non-inhibitory form [36]. Binding to vitronectin stabilizes the active form, and the fraction of active PAI-1 in plasma has been reported to vary between 20 and 90 percent [75-77]. The interreaction between t-PA and PAI-1 is rapid, with a second order rate constant of approximately 10^7 M^{-1} s^{-1} [24] and there is a several molar excess of PAI-1 over t-PA in plasma [78]. The cellular origin of circulating PAI-1 is uncertain, but *in vitro* PAI-1 can be synthesized by a variety of cell types, including endothelial cells, smooth muscle cells, macrophages, hepatocytes, adipocytes and platelets. Although about 90% of circulating PAI-1 is found in platelets, platelet PAI-1 is not considered to contribute to plasma PAI-1 [75], but may play an important role during a thrombotic event [79]. In contrast to t-PA, PAI-1 is not stored in endothelial cells and consequently there is no regulated release of PAI-1 from the endothelium. A number of factors including lipoproteins, glucose, cytokines, thrombin, growth factors, insulin and other hormones have been found to regulate the expression of PAI-1 [80]. *In vivo*, elevated plasma levels of PAI-1 is a common feature of the insulin resistance syndrome, and show correlations with *e.g.* obesisty, hyperlipidemia, hyperinsulinemia, and hypertension [81, 82].

Impaired fibrinolytic response

As stated before, our group has previously reported on a reduced capacity for acute t-PA release in hypertensive subjects [4, 5]. The underlying mechanism of this impairment is unclear, but studies from our laboratory indicate that at least a part of the defective response could be explained by an increased pressure load on the endothelium [3]. Thus, it is likely that the impaired t-PA release is correlated to a disturbed haemodynamic milieu in the vessel. Other factors potentially contributing to a defect t-PA response might be inflammatory molecules. Several proatherothrombotic conditions are associated with a low-grade inflammation and increased circulating levels of pro-inflammatory cytokines are frequently observed in patients with *e.g.* atherosclerosis, obesity, diabetes, and hypertension [83].

Haemodynamic forces

Due to its position in the vasculature, the endothelium is a target for biomechanical forces imposed on the vessel wall by intraluminal pressure and the friction of flowing blood. The haemodynamic forces acting on the vessel wall can be described by two major components: a) tensile stress, which is created by the blood pressure and is the elongation the cell undergoes from the rhytmic distension of the vessel and b) shear stress, which is the frictional force imposed on the endothelial cell surface by the flowing blood [84, 85] (Figure 2).

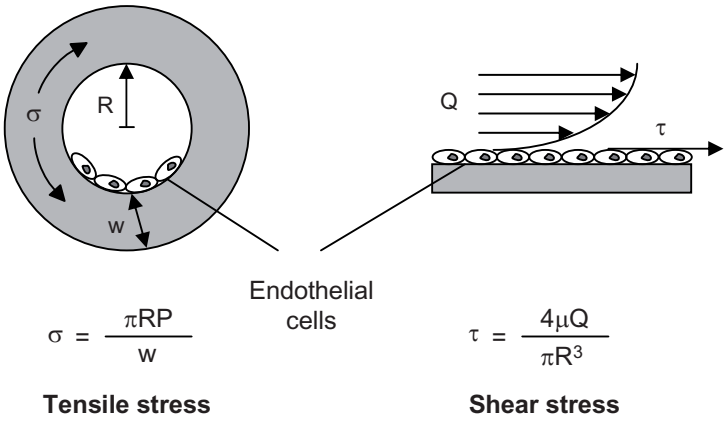

Figure 2. Haemodynamic forces acting on the blood vessel wall. The tensile stress (σ) is generated by the blood pressure, has a circumferential distribution, and affects all constituents of the vessel wall. It is proportional to the transmural pressure (P) and radius (R) and inversely proportional to the vessel wall thickness (w). Fluid shear stress (τ) is a frictional force exerted by the blood flow and it acts exclusively on the endothelium. It is proportional to the blood flow (Q) and viscosity of the blood (μ) and inversely proportional to the third power of the radius.

In vivo observations suggest that haemodynamic forces can alter vascular structure and function. This capacity is illustrated for instance by atherosclerotic lesions, which preferentially localize close to vascular branch points and bifurcations, where the flow profile is nonuniform and disturbed [86, 87]. Another example is provided by remodeling processes, which are induced by altered mechanical forces and aim to restore basal levels of tensile stress and shear stress [88, 89]. Acute changes in stretch or shear stress correlate with transient adjustments in vessel diameter, mediated through the release of vasoactive agonists or change in myogenic tone, while chronic alterations usually instigate important adaptive alterations of vessel wall shape and composition. Shear stress varies in the vascular tree from a few dyn/cm^2 in veins to 2-40 dyn/cm^2 (locally up to 100 dyn/cm^2) in arteries [6, 84, 90, 91]. Typically, shear stress in the arterial network is actively regulated at a constant level of approximately 15 dyn/cm^2 [84, 85, 92]. Tensile stress, or strain, is in large arteries reported to range from 2 to 18% during the normal cardiac cycle [93].

Mechanical forces can initiate complex signal transduction cascades leading to functional changes within the cell, often triggered by activation of integrins, but also by stimulation of other structures such as G-protein receptors, tyrosine kinase receptors, ion channels or junction proteins [94-96]. The stimulated mechanosensors activate a complex array of second messengers, including PKC, phosphatidylinositol 3-kinase/Akt, focal adhesion kinase and mitogen-activated protein kinases (MAPK), which transduce the signals to the nucleus and activate transcription factors [94-96]. The MAPK cascade is an important pathway whereby signals originating from mechanical forces can lead to gene expression and protein synthesis [97]. This pathway implicates the sequential phosphorylation and activation of cytoplasmic protein kinases MEKK, MEK, and finally MAPK. The MAPK cascade comprises in reality several different pathways; the c-jun N-terminal kinase (JNK), the p38 MAPK, the extracellular signal-regulated kinase 1/2 (ERK1/2) and the big MAPK-1 (BMK-1/ERK5) pathways [98], which are triggered in response to various stimuli and initiate distinct cellular responses. Generally, JNK and p38 MAPK play important roles in inflammatory and stress responses, while ERK1/2 and BMK-1 are primarily involved in growth and cytoprotective functions [99].

Shear stress and mechanical strain share many signaling pathways by which they mediate their effects, although differences exist, *e.g.* the time course of the responses. The same holds true for transcription factors activated by the two mechanical forces. AP-1, CREB, nuclear factor (NF)-κB, Sp1 and early growth response (Egr)-1 are all activated in response to both shear and tensile stress [100-105], several of which are also downstream effector molecules of the MAPK cascades.

The influence of biomechanical forces on regulation of endothelial function has been extensively studied in different *in vitro* experimental setups. However, not many reports have dealt with t-PA and the scarce amount of data on this topic have shown somewhat inconsistent results. Moreover, few studies have covered both short and long-term effects, and little attention has been devoted to the regulation of t-PA gene expression. Regarding strain, t-PA secretion has both been reported to be enhanced [106] and unaffected by this stimuli [107, 108]. A single study has reported on t-PA

mRNA, presenting a stimulatory effect of strain [109]. Available literature on shear regulation of t-PA have reported enhanced protein secretion and gene expression following stimulation with physiological levels of laminar shear stress [110-114].

Inflammation

Inflammatory mechanisms shift the haemostatic balance to favour the activation of coagulation and, in the extremes, either disseminated intravascular coagulation or thrombosis. Inflammatory mediators can elevate platelet count, platelet reactivity, downregulate natural anticoagulants mechanisms, initiate the coagulation system, facilitate propagation of the coagulant response and impair fibrinolysis [115]. Increased levels of the inflammatory marker C-reactive protein (CRP) are predictive of myocardial infarction and stroke [83], and more specifically, patients with chronic inflammatory diseases such as rheumatoid arthritis and systemic lupus erythematosus have an increased risk of developing these disorders [116, 117]. The observation that inhibition of tumor necrosis factor (TNF)-α reduces the incidence of cardiovascular events [118], suggests that the effect is at least partly mediated by proinflammatory cytokines. TNF-α is the prototypic proinflammatory cytokine and acts as one of the most important promoters of inflammation. It is principally derived from mononuclear phagocytes and endothelial cells are a major cellular target of its actions [119]. Exposure of endothelial cells to TNF-α results in activation of three major proinflammatory signaling pathways; the NF-κB pathway, the p38 MAPK pathway, and the JNK pathway [120, 121]. These signaling cascades interact through a complex network, which mediate gene regulatory effects primarily by activation of the two transcription factors NF-κB and AP-1 [121, 122].

In resting cells NF-κB is retained in the cytoplasm because of its association with inhibitor proteins (IκBs). Binding of TNF-α to its receptors results in phosphorylation of IκB by the IκB kinase (IKK) complex, which target the protein for rapid ubiquitination and degradation [120]. Degradation of IκB unmaskes a nuclear localization sequence of NF-κB making it free to translocate to the nucleus and regulate transcription. Once in the nucleus the NF-κB transcriptional activity can be modulated further through phosphorylation by various protein kinases. In endothelial cells, TNF-α induced NF-κB is formed of homo- or heterodimers involving p50, p65 and c-Rel subunits [120]. AP-1, on the other hand, is a heterogenous collection of dimeric transcription factors comprising Jun, Fos and ATF subunits [123], and is in response to TNF-α an outcome of preferentially JNK and p38 MAPK signaling [122, 124].

Proinflammatory cytokines, like TNF-α and interleukin (IL)-1β, have been reported to suppress endothelial fibrinolytic activity, most evidently reflected by an elevated expression of PAI-1 [125-128]. The literature is, however, somewhat conflicting regarding the effects of proinflammatory cytokines on the expression of t-PA, with most *in vitro* data supporting a suppressive effect on t-PA expression [125, 127, 129], in contrast to *in vivo* observations reporting stimulatory effects [130, 131].

AIMS

Against this background the objective of the present work was:

- to investigate if the impaired fibrinolytic capacity observed in hypertensive patients could be reversed by antihypertensive therapy (Paper I).

- to test the hypothesis that the same impairment could be due to an enhanced tensile stress (Paper II) and/or an enhanced shear stress (Paper III) acting on the endothelium.

- to test the hypothesis that proinflammatory cytokines, like TNF-α, suppress endothelial t-PA expression, and if so, to elucidate the underlying mechanisms behind this response (Paper IV).

MATERIAL AND METHODS

Subjects

Study I included 20 white subjects (age, 61 years; range, 39 to 75 years; 12 men and 8 women) with documented primary hypertension. All of the subjects were nonsmokers without a history of diabetes mellitus or other major illness and were on no other medication than antihypertensive drugs. All of the women were postmenopausal. Patients with secondary hypertension, blood lipid derangements or impaired glucose tolerance were excluded. After study day 1, patients were randomized to open treatment with either the angiotensin-converting enzyme (ACE) inhibitor lisinopril or the calcium channel blocker felodipine. The patients in the lisinopril group had, on the average, a longer duration of hypertension of 15 years compared with 5 years in the felodipine group. The severity of hypertension was similar in the 2 groups, with 3 and 2 patients, respectively, on dual therapy on enrollment in the felodipine and lisinopril group. Three subjects in the felodipine group and 1 subject in the lisinopril group were previously untreated. The remaining patients were on monotherapy. In the felodipine group, 3 patients had been treated previously by ACE-inhibitors and 2 patients by calcium channel blockers. In the lisinopril group, 5 patients previously had an ACE inhibitor, and 2 had a calcium channel blocker. A smaller number of subjects in both groups were treated with β blockers, angiotensin II receptor blockers, and diuretics.

Table 1. Baseline characteristics of patients in Study I.

Parameter	Felodipine group	Lisinopril group	p value
Age, y	62.4 (2.0)	60.5 (3.5)	n.s.
Sex (male/female)	6/4	6/4	
Body mass index, kg/m^2	25.7 (0.9)	26.4 (0.9)	n.s.
Waist/hip ratio	0.91 (0.02)	0.92 (0.2)	n.s.
Total cholesterol, mmol/L	5.2 (0.3)	5.6 (0.1)	n.s.
Triglycerides, mmol/L	1.4 (0.1)	1.5 (0.1)	n.s.
Plasma glucose, mmol/L	5.5 (0.2)	5.5 (0.1)	n.s.
Plasma insulin, mU/L	8.3 (1.3)	8.5 (1.4)	n.s.

Data are mean and SEM. n.s. indicates not significant.

Cell culture

The experiments presented in Study II-IV were carried out on cultured human aortic endothelial cells (HAEC) and human umbilical vein endothelial cells (HUVEC). HAECs were purchased from Clonetics, while HUVECs were isolated from fresh umbilical cords obtained from normal deliveries at the maternity ward of the hospital. HUVECs were prepared by collagenase digestion according to the method of Jaffe *et al.* [132]. In brief, the umbilical vein was catheterized under sterile conditions and

the blood was removed by infusion of warm phosphate buffer saline (PBS). Endothelial cells were explanted by incubation with 0.1% collagenase following gentle manipulation of the umbilical cord. Isolated cells were maintained in EGM-2 complete culture medium, consisting of EBM-2 basal medium (Clonetics) supplemented with 2% fetal bovine serum and growth factors (SingleQuots® kit; Clonetics) in plastic culture flasks at 37°C in a humidified 5% CO_2 incubator. The medium was replaced every 2-3 days and sub-cultures were obtained by trypsin/EDTA treatment of confluent monolayers. HUVECs and HAECs were used in experiments at passage 1 and 4-6, respectively.

Experimental design

Study I

Study I was designed to explore if the impaired fibrinolytic capacity in hypertensive subjects could be restored by lowering the blood pressure. Studies began ≈4 weeks after cessation of antihypertensive treatment, and patients with blood pressure levels >140 mm Hg systolic and 90 mm Hg diastolic were included. After a baseline examination including 24 h blood pressure monitoring, patients went through an invasive perfused-forearm study to determine the capacity for stimulated t-PA release and endothelium-dependent vasodilation. Thereafter, patients were randomized to open treatment with either lisinopril (Zestril) at 10 mg or felodipine (Plendil) at 5 mg daily. The dosage was individually titrated to approach target levels of blood pressure (130/85 mm Hg) or, if this was not achieved, to a maximal dose of lisinopril at 20 mg x 2 or felodipine at 10 mg x 2 daily. After ≥8 weeks of treatment on target levels or with maximal drug dose, a second perfused-forearm study was performed according to an identical protocol as the first examination.

The perfused-forearm study

Local t-PA release rate was assessed by the perfused-forearm model [43, 47, 51]. The subject attended the laboratory after an overnight fast. On the second experimental day, the patients took their study medication in the morning. An 18-gauge arterial polyethylene catheter was introduced percutaneously with modified Seldinger technique into the brachial artery of the nondominant arm. An indwelling cannula was placed retrogradely into a deep ipsilateral antecubital vein. Intraarterial blood pressure was recorded by an electrical transducer connected to an SC 9000 monitor (Siemens Medical Systems Inc). After each venous blood sampling, forearm blood flow (FBF) was assessed by venous occlusion plethysmography with a mercury-in-silastic strain gauge. Means of 3 to 5 recordings were expressed in mL per min and volume of tissue.

After cannulation of the artery, ≥45 minutes were allowed before baseline recordings (15 min). Thereafter, substance P (CLINALFA) at 8 pmol/mL was infused for 20 min into the brachial artery at a constant rate of 1 mL/min. Postinfusion recordings were performed for 20 min. During preinfusion and postinfusion baseline periods, blood samples were collected simultaneously from the brachial artery and vein. During substance P infusion, venous blood samples were obtained at 1.5, 3, 6, 9, 12, 15, and 18

min. To avoid interruption of the infusion, arterial blood was obtained only at baseline and at the end of the infusion and in-between values interpolated from these values. Blood was collected in chilled tubes containing 0.1 vol of 0.45 mol/L sodium citrate buffer (pH 4.3; Stabilyte, Biopool) for determination of fibrinolytic proteins. Plasma was isolated by centrifugation and thereafter immediately frozen and stored at –70°C until assay. Plasma concentrations of t-PA and PAI-1 antigen were determined with ELISA, while active t-PA and PAI-1 were analyzed by biofunctional immunosorbent assays.

Calculations

Net release or uptake rate of fibrinolytic factors was calculated according to the formula:

$$\text{Net release/uptake rate} = (C_V\text{-}C_A) \times FPF$$

where C_V denotes venous, and C_A arterial plasma concentration. FBF was interconverted to forearm plasma flow (FPF) by haematocrit. Total cumulative t-PA release in response to substance P was estimated for each individual as area under the curve from baseline until 20 minutes after terminating the infusion. Forearm vascular resistance (FVR) was calculated as the ratio of mean arterial pressure to FBF and expressed in arbitrary resistance units.

Study II

This study was designed to evaluate the impact of tensile stress on regulation of the fibrinolytic enzymes t-PA, u-PA and PAI-1. Cyclic strain experiments were performed with a Flexercell Tension Plus FX-4000T system (Flexcell International Corporation) equipped with a 25-mm BioFlex loading station designed to provide a well-defined equibiaxial and circumferential strain across a membrane surface (Figure 3). BioFlex loading station is composed of a single plate and six planar 25-mm cylinders per plate centered beneath each well of the BioFlex plate, and the top surface is just below the BioFlex membrane surface. Each BioFlex membrane is stretched over the post when under vacuum pressure, creating a single-plane uniformly stretched circle. Both static HAEC cultures and cells exposed to cyclic strain were seeded onto identical collagen coated Bioflex plates to ensure standard culture conditions. Cells were grown to confluence and medium (EGM-2) was exchanged in all plates prior to start of the mechanical deformation experiments and thereafter every 24 h. Strain stimulation was set to 10% stretch at 60 cycles/min (0.5 s elongation alternating with 0.5 s relaxation). All cell batches were exposed to strain for 6, 24, 48 and 72 h and mechanically stimulated cells were compared with static control cells from the same individual, within the same experiment and from the same time-point. Real-time RT-PCR and ELISA was used to evaluate effects on mRNA expression and protein secretion of t-PA, u-PA and PAI-1.

Study III

Study III was designed to investigate how, and by which mechanisms, shear stress regulates t-PA gene expression. Flow studies were performed with the Streamer™

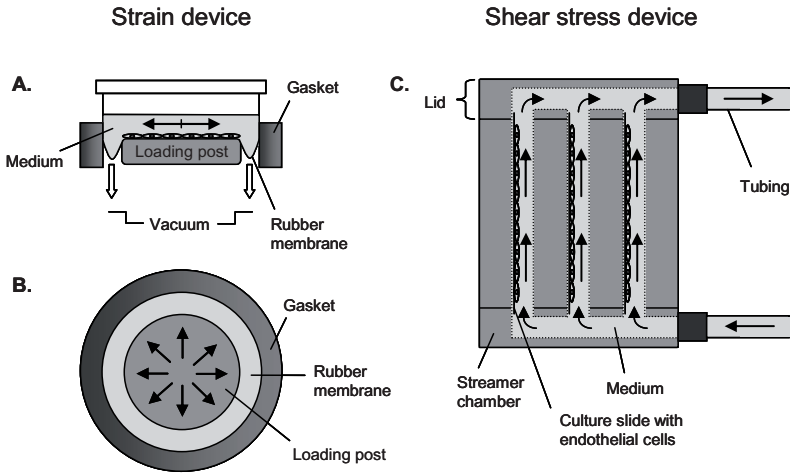

Figure 3. Schematic of the strain and shear stress devices used in Study II and III, respectively. **A.** Sideview of BioFlex well in the strain device. The rubber membrane is stretched over the loading post when under vaccum pressure. Cells covering the loading post are exposed to equibiaxial and circumferential strain. **B**. BioFlex well viewed from above. **C**. Cross-section diagram of the Streamer shear stress chamber. Cells on applied culture slides are exposed to steady laminar shear stress by the recirculated medium.

shear stress device (Flexcell) (Figure 3). The device is a parallel-plate flow system that enables stimulation of cultured cells with fluid-induced steady laminar shear stress. Endothelial cells were seeded onto fibronectin coated (Roche Diagnostics) culture slides (Flexcell) and grown to confluence. On day of experiment culture slides were mounted in two Streamer chambers for simultaneous stimulation with either low (1.5 dyn/cm^2) or high (25 dyn/cm^2) laminar shear stress. Each Streamer chamber was incorporated into a recirculating loop fed with perfusion medium (50% EGM-2, 50% M199, total 2% FBS) from a shared medium reservoir. Medium was driven by peristaltic roller pumps and each loop included a pulse dampener to ensure steady laminar flow. The various components were connected by silicone rubber tubing and both systems were placed in a 37°C humidified 5% CO_2 incubator. Control slides with static endothelial cells were placed in the same cell culture incubator. The experiments in this study were performed on HUVECs and HAECs and lasted for up to 24 h. Influence of shear stress on t-PA and PAI-1 mRNA expression, activation of the NF-κB and MAPK cascades, and nuclear protein interactions with gene regulatory elements in the t-PA promoter were assessed by real-time RT-PCR, Western blotting and EMSA, respectively.

Study IV

This study aimed at elucidating the underlying mechanisms of TNF-α regulated t-PA gene expression. HUVECs were seeded in plastic culture plates or plastic culture flasks and grown to confluence. On the day of experiment, cells were added fresh EGM-2

culture medium with or without signaling pathway inhibitors. Cells were thereafter placed in a cell culture incubator for 1 h before stimulation with 0.1-10 ng/ml of human recombinant TNF-α (Sigma-Aldrich) for up to 48 h. Inhibitors were present during the whole experiments. Six μM parthenolide (Sigma-Aldrich) was used to inhibit NF-κB signaling, 25 μM SB203580 (Biosource) to inhibit p38 MAPK, and 10 μM SP600125 (Calbiochem) to inhibit JNK. Regulation of t-PA mRNA expression was analyzed by real-time RT-PCR and interactions between nuclear proteins and regulatory elements in the t-PA promoter with EMSA. ELISA was used to confirm that observed effects were relevant also on the level of t-PA secretion.

Analyzing techniques

Enzyme-linked immunosorbent assay (ELISA)

The plasma levels of t-PA and PAI-1 antigen (Study I), the concentration of t-PA, PAI-1 and u-PA antigen in the cell culture medium (Study II and IV), and intra-cellular levels of t-PA and u-PA antigen (Study II) were determined with ELISA (TintElize t-PA, Biopool International; COALIZA PAI-1, Chromogenix and ZYMOTEST u-PA, Haemochrom Diagnostica). The principle of these assays is that the samples, or a standard containing human recombinant protein, are added to microtest wells that are coated with anti-t-PA/PAI-1/u-PA IgG. After t-PA/PAI-1/u-PA has been allowed to bind to the antibodies, peroxidase-labeled anti-t-PA/PAI-1/u-PA IgG is added. Wells are washed to remove unbound antibodies and peroxidase substrate is added. Peroxidase then converts the substrate to a yellow product that is directly proportional to the amount of protein present in the sample. All samples were assayed in duplicate. Plasma t-PA and PAI-1 activity (Study I), *i.e*. the free, uncomplexed fraction of respective enzyme, was measured by biofunctional immunosorbent assays (Chromolize t-PA and Chromolize PAI-1, Biopool International) and expressed in ng/ml using the specific activity of 0.60 and 0.75 IU/ng, respectively (data on file, Biopool International). Intraassay variation coefficients were <5% for all assays.

Real-Time RT-PCR

Following experiments in which endothelial cells had been stimulated with strain, shear stress or TNF-α (Study II-IV), total RNA was isolated using either Trizol (Invitrogen, Study II) or RNeasy Mini Kit (Qiagen, Study III and IV). Contaminations of DNA were removed by treatment with DNase (Ambion or Qiagen, respectively). RNA concentrations and purity were determined with absorbance measures at 260/280 nm wavelength and RNA quality was controlled on 1% agarose gel. mRNA was converted to cDNA with GeneAmp RNA PCR kit (Applied Biosystems).

Levels of t-PA, u-PA, PAI-1, VCAM-1, ICAM-1 and eNOS mRNA were analyzed with real-time RT-PCR, performed on a ABI Prism 7700 Sequence Detection System (Applied Biosystems), and normalized relative to the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH is a constititively expressed gene, and thus works as an internal standard to correct for potential variation in RNA loading, cDNA synthesis, or efficiency of PCR amplification. The principle of the assay is that when a fluorescently labeled probe is hybridized to its target sequence during

PCR, the Taq polymerase cleaves the reporter dye from the non-extendable probe. The reporter dye is then released to solution and the increase in dye emission is monitored in real-time. The threshold cycle (C_T) is defined as the cycle number at which the reporter fluorescence reaches a fixed threshold level. There is a linear relationship between C_T and the log of initial target copy number as shown by Higuchi *et al*. [133]. Relative quantification of gene expression was analyzed as a treatment-to-control expression ratio using the comparative C_T method (User Bulletin #2, Applied Biosystems). The relative expression value of the target gene is obtained by calculating the difference in threshold cycles for a target and a reference gene in a treated sample, and comparing it to that of a control sample.

Oligonucleotide primers and Taqman® probes for quantification of t-PA, PAI-1, eNOS and GAPDH mRNA were designed from the GenBank database using Primer Express version 1.5 (Applied Biosystems), whereas u-PA mRNA was quantified with Taqman® Assays-by-Demand™ and VCAM-1 and ICAM-1 mRNA with Taqman® Assays-by-Design™ (Applied Biosystems) (oligonucleotide sequences in Table 2).

Table 2. Primers and probes used in real-time RT-PCR

Oligonucleotide
t-PA
FP: 5´-GGC CTT GTC TCC TTT CTA TTC G-3´
RP: 5´-AGC GGC TGG ATG GGT ACA G-3´
PR: 5´-TGA CAT GAG CCT CCT TCA GCC GCT-3´
PAI-1
FP: 5´-GGC TGA CTT CAC GAG TCT TTC A-3´
RP: 5´-TTC ACT TTC TGC AGC GCC T-3´
PR: 5´-ACC AAG AGC CTC TCC ACG TCG CG-3´
VCAM-1
FP: 5´-GGA AGA AGC AGA AAG GAA GTG GAA T-3´
RP: 5´-GAC ACT CTC AGA AGG AAA AGC TGT A-3´
PR: 5´-CCA AGT TAC TCC AAA AGA C-3´
ICAM-1
FP: 5´-CCA GGA GAC ACT GCA GAC A-3´
RP: 5´-TGG CTT CGT CAG AAT CAC GTT-3´
PR: 5´-ACC ATC TAC AGC TTT CC-3´
eNOS
FP: 5´-CGC AGC GCC GTG AAG-3´
RP: 5´-ACC ACG TCA TAC TCA TCC ATA CAC-3´
PR: 5´-CCT CGC TCA TGG GCA CGG TG-3´
GAPDH
FP: 5´-CCA CAT CGC TCA GAC ACC AT-3´
RP: 5´-CCA GGC GCC CAA TAC G-3´
PR: 5´-AAG GTG AAG GTC GGA GTC AAC GGA TTT G-3´
u-PA
Sequences not provided by Applied Biosystems.
ID: Hs00170182_m1

Abbreviations: FP denotes forward primer, RP reverse primer and PR probe.

Primer pairs were selected so the amplicon spanned an exon junction to avoid amplification of genomic DNA. All probes were dual-labeled with 5´-reporter dye FAM (6-carboxy-fluorescein) and 3´-quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine). cDNA from 30 ng of total RNA, Taqman® Universal PCR Mastermix (Applied Biosystems), 10 pmol of each primer and 5 pmol probe (1.25 μL 20X Assays-by-Demand or 0.42 μL 60X Assays-by-Design mix for u-PA, VCAM-1 and ICAM-1) were mixed for each reaction in a final volume of 25 μL. Samples were amplified in duplicate.

Western blotting

Effects of shear stress or TNF-α on the activation of the NF-κB, ERK1/2, p38 MAPK and JNK pathways were evaluated by Western blotting (Study III and IV). Stimulated HUVECs were harvested in Laemli sample buffer (Bio-Rad) with 5% β-merkaptoethanol, sonicated and boiled before being applied to a 10% Tris-Glycine gel (Cambrex) and electrophoresed in 1 X running buffer (Bio-Rad). Resolved proteins were transferred by blotting onto Hybond-P polyvinylidene fluoride membranes (Amersham Biosciences) in transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, 20% methanol and 0.01% SDS). To minimize nonspecific binding, membranes were placed in blocking solution (5% fat-free dried milk in tris-buffered saline with 0.05% Tween-20 (TBST)) for 1 h. Thereafter, membranes were incubated over night, 4°C, with primary antibodies (Cell Signaling) directed against the phosphorylated or total forms of p65 (NF-κB subunit), ERK1/2, p38 MAPK and JNK, 1:1000 in TBST supplemented with 5% bovine albumin, and thereafter with secondary antibody (anti-rabbit IgG, horseradish peroxidase linked, 1:2000 in blocking solution for 1 h at room temperature. Proteins were visualized using SuperSignal Chemiluminescent Substrate (Pierce Biotechnology).

Electrophoretic mobility shift assay (EMSA)

EMSA was used to detect interactions between nuclear proteins and gene regulatory elements in the t-PA promoter (Study III and IV). Five double-stranded oligomers, each designed to contain a t-PA promoter specific element of interest, and consensus oligonucleotides for NF-κB and AP-1 (Promega) were used as EMSA probes. The t-PA specific elements were; the recently described functional κB element found in the t-PA gene of human neuronal cells [72] (5´-agggccggggattcccagtcta-3´), the t-PA cyclic adenosine monophosphate (cAMP) response element (CRE)-like site [69] (5'-attcaatgacatcacggctgtg-3´), the t-PA shear stress responsive element (SSRE) [73] (5´-caaggtctggtctcagccagacat-3´), and the t-PA GC boxes II and III [70] (5´-acacagaaacccgcccagccgg-3´ and 5´-accgaccccacccccctgcctgg-3´, respectively). To identify specific proteins involved in DNA-binding, supershift experiments were performed using antibodies (Santa Cruz) against HUVEC-expressed subunits of the NF-κB complex [121]; p50, p65 and c-Rel, and against HUVEC t-PACRE binding proteins [69]; CREB, ATF-2 and c-jun.

The preparation of nuclear extracts from HUVECs was performed as previously described [134] and protein concentrations were quantified with a fluorometer (FLUOstar Optima; BMG LabTechnologies) using Bio-Rad reagents. Labeling of the oligo-

mers was carried out as described using T4 polynucleotide kinase and [γ-^{32}P]ATP [135]. Annealing was performed (excluded step for consensus oligonucleotides) by adding a molar excess of the complementary strand to the kinase-treated, heat-inactivated mixture, which was subsequently heated to 95°C, after which the samples were left to anneal during the cooling-down process. Probes were gel-purified by electrophoresis through 12% native polyacrylamide gels, visualized by autoradiography, excised and eluted overnight at 37°C in buffer containing 0.5 M ammonium acetate and 1 mM EDTA. Supernatant solutions containing the labeled oligomer were precipitated with ethanol in the presence of tRNA and resuspended in NaCl/Tris/EDTA buffer to approximately 1000 cps/μL as described [71]. Binding reactions were carried out in a volume of 10 μL containing 5 μg crude nuclear extract in 2 μL Osborne buffer D [134], 1 μg poly[d(I-C)] [polydeoxy(inosinate-cytidylate)], 3 μL SMK buffer (12 mM spermidine, 12 mM $MgCl_2$, and 200 mM KCl) and ^{32}P-labeled probe (4 μL; 100 cps diluted in buffer D) as described (Study IV). The binding reactions were analyzed by electrophoresis in a 5% native polyacrylamide gel, and visualized by autoradiography.

Statistics

In Study I, between-treatment status comparisons of single variables were performed by Student *t*-test. Responses to substance P were evaluated by 2-way (treatment/no treatment and time) and 1-way (time) ANOVA for repeated measures. Between-drug comparisons of responses were performed by 2-way ANOVA during treatment (drug and time). Proportions of categorical data were compared by χ^2 test. The statistical evaluation in Study II was performed using 2-way ANOVA. When ANOVA indicated a significant treatment or treatment x time effect, responses at individual time points were evaluated by contrast analysis. Paired Student *t*-test was used to evaluate statistical differences in Study III and IV. Values are throughout each study presented as mean and SEM. Findings were considered significant at $p<0.05$.

RESULTS

Study I

Baseline characteristics

Baseline haemodynamic and fibrinolytic variables are shown in Table 3. After target blood pressure levels were reached, the patients were treated on average for 10 and 9 weeks in the lisinopril and felodipine groups, respectively, before the second study day was performed. Treatment lowered the intraarterial systolic and diastolic blood pressure on the average from 165(3)/82(2) to 140(3)/71(1) mm Hg ($p<0.01$ throughout). Changes in blood pressure were similar in the lisinopril and felodipine groups, or 24/12 and 26/10 mm Hg, respectively. Baseline FBF and FVR were not affected by treatment. Also, baseline concentrations of the fibrinolytic variables did not change significantly in either group by treatment.

Table 3. Summary of baseline haemodynamic, inflammatoric, and fibrinolytic variables

Parameter	Untreated	Treated	p value
Systolic blood pressure,* mm Hg	165.4 (3.0)	140.3 (3.4)	<0.00001
Diastolic blood pressure,* mm Hg	81.9 (1.5)	71.1 (1.4)	<0.00001
Mean arterial pressure,* mm Hg	115.3 (1.7)	98.7 (1.9)	<0.00001
Forearm blood flow, mL/L tissue	62.3 (7.2)	47.7 (4.8)	n.s.
Forearm vascular resistance, arbitrary units	2.5 (0.3)	2.6 (0.3)	n.s.
Plasma t-PA antigen, ng/ml	8.9 (0.5)	8.6 (0.6)	n.s.
Plasma t-PA activity, IU/ml	0.66 (0.05)	0.68 (0.05)	n.s.
Plasma PAI-1 antigen, ng/ml	31.1 (3.2)	29.8 (6.2)	n.s.
Plasma PAI-1 activity, IU/ml	6.1 (1.1)	6.7 (2.5)	n.s.
Hs-CRP, mg/L	3.6 (0.4)	2.7 (0.5)	n.s.

Data are mean and SEM. n.s indicates not significant; Hs-CRP, high-sensitive C-reactive protein.
*Blood pressure was measured intraarterially at the beginning of the experiment.

Antihypertensive therapy restores impaired t-PA response

Substance P induced highly significant t-PA secretory responses of the forearm, both when patients were untreated and when they were on active antihypertensive treatment (ANOVA, $p<0.0001$). In line with the hypothesis, the t-PA antigen release response was significantly greater on treatment (2-way ANOVA, $p=0.0001$) (Figure 4A). There were no significant differences in the t-PA release responses between the treatment groups (Figure 4B). The cumulated t-PA antigen release during substance P infusion

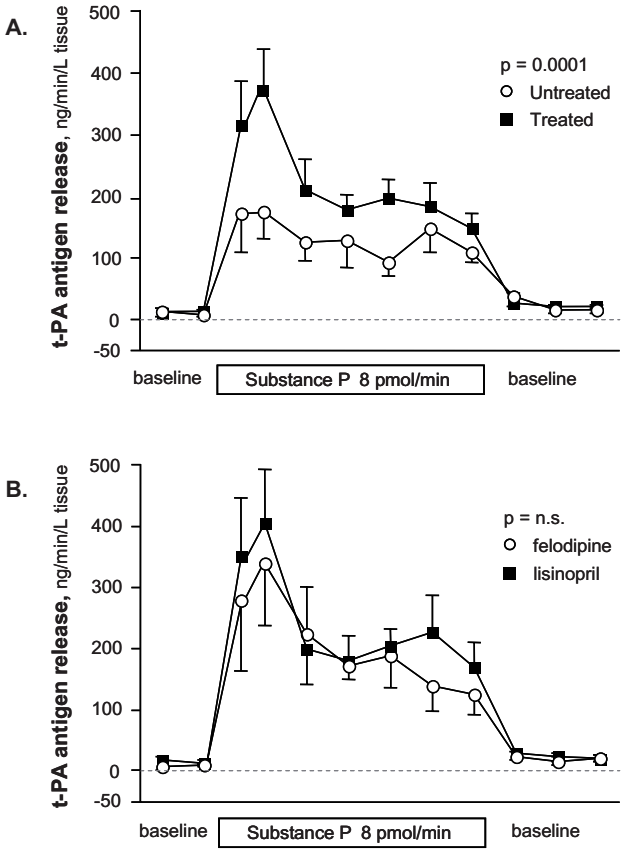

Figure 4. Antihypertensive therapy restores impaired t-PA response. Net forearm release rates of t-PA antigen during baseline and in response to 20 min of intraarterial infusion of substance P (8 pmol/min) (baseline measurements 15 min before and 20 min after the infusion) **A.** untreated compared to treated hypertensive patients **B.** felodipine compared to lisinopril treated hypertensive patients.

increased from 3000 (655) to 4557 (701) ng/L tissue with treatment ($p<0.05$). The release of active t-PA during the first 6 minutes of infusion was significantly improved by treatment (2-way ANOVA, $p=0.03$).

The t-PA antigen release, which was in the order of 9.5 and 11.8 ng/min and L tissue at baseline, increased significantly and peaked at 257 (58) and 445 (77) ng/min and L tissue during the substance P infusion, in untreated and treated patients, respectively ($p<0.0001$ for both). The peak t-PA release was significantly improved by treatment and was of almost identical magnitude in the lisinopril and felodipine groups. On the whole, substance P induced a 27- and 38-fold increase in t-PA release in untreated

and treated patients, respectively ($p<0.05$ for change in fold increase). Antihypertensive treatment also altered the temporal response pattern to stimulation (Figure 5). When patients were untreated, one-third of the patients had a delayed onset of the t-PA response; in 6 of 20 patients, the peak release rate occurred 9 minutes or later after initiating substance P stimulation. The response pattern was normalized with treatment, and on the second study day, all of the patients had the peak release rate during the first 6 minutes of stimulation (χ^2 test, $p=0.008$). Thus, treatment improved the response pattern and shortened the average time to peak secretion from 6.7 (1.4) to 2.7 (0.3) min ($p=0.01$).

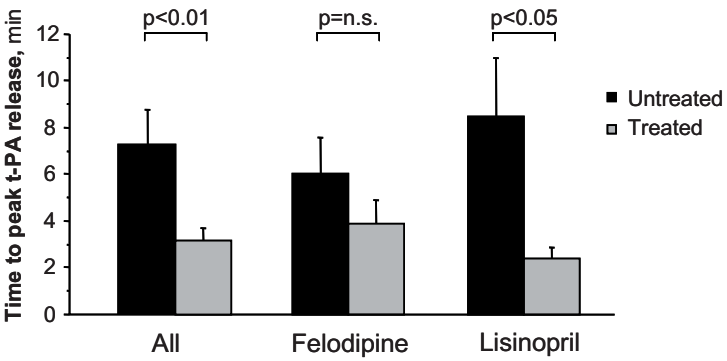

Figure 5. Antihypertensive treatment improves the time (in minutes) to peak t-PA release. Black and grey bars represent untreated and treated hypertensive patients, respectively. Left, the whole group; middle, felodipine-treated group; and right, lisinopril-treated group.

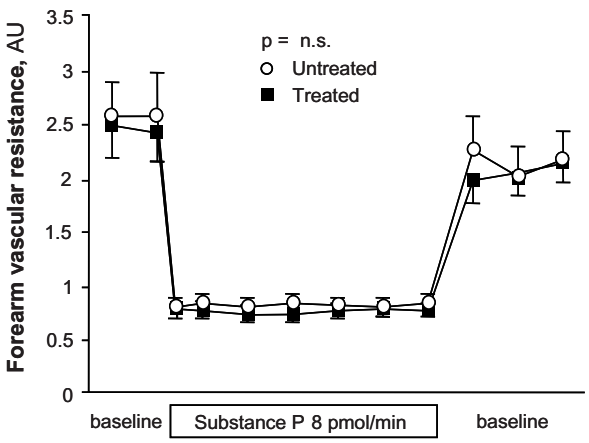

Figure 6. Treatment does not affect vasodilator responses. Forearm vascular resistance (arbitrary units) during baseline and in response to 20 min of intraarterial infusion of substance P (8 pmol/min) in untreated and treated hypertensive patients (baseline measurements 15 min before and 20 min after the infusion).

Treatment does not affect vasodilator responses

Substance P induced highly significant decreases in FVR and increases in FBF, both when patients were untreated and on active treatment (ANOVA, $p<0.0001$ for all). The responses of FVR (Figure 6) and FBF to substance P stimulation were of the same magnitude on both treatment days.

Study II

Cyclic strain suppresses expression of t-PA

Tensile stress was applied to cultured endothelial cells in order to outline the impact of this particular haemodynamic force on regulation of fibrinolytic enzymes. In response to cyclic strain, t-PA gene expression showed a biphasic temporal response pattern with an early transient inductive response at 6 h, which switched to a suppression during prolonged strain stimulation reaching a plateau phase at 48 and 72 h (ANOVA treatment x time: $p<0.001$) (Figure 7). The transient increase in t-PA mRNA expression declined below baseline at 24 h and was significantly suppressed by 48 and 72 h of cyclic strain stimulation. The reduction was approximately 30% in comparison to static control cells ($p<0.01$). A similar pattern was observed for the t-PA secretion over 72 h, with the exception that the strain effect was more delayed (ANOVA treatment x time: $p<0.01$). In parallel with t-PA mRNA data, the induction peaked at 6 h and thereafter succesively diminished by prolonged cyclic strain stimulation and from 48 to 72 h switched from an induction to a 12% reduction. Interestingly, strain was observed to regulate gene expression of the other plasminogen activator, u-PA, in a similar mode. The difference was that the strain mediated suppression of u-PA mRNA was present first after 72 h of stimulation (-19%, $p<0.01$).

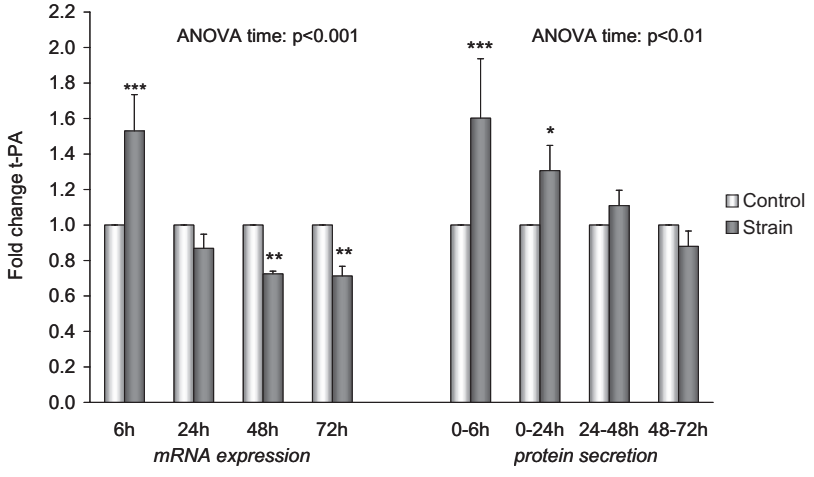

Figure 7. Prolonged cyclic strain suppresses t-PA mRNA expression and protein secretion. HAECs were exposed to 10% cyclic strain for 6-72 h and analyzed by real-time RT-PCR and ELISA for alterations in t-PA gene and protein expression, respectively. Results are expressed as fold change compared to static control cells. n=4, *p<0.05, **p<0.01 and ***p<0.001.

Cyclic strain induces expression of PAI-1

Analyses of PAI-1 gene expression in the same samples showed increased expression in strained HAECs at all time-points (ANOVA treatment: $p<0.05$) (Figure 8). The magnitude of the relative increases in PAI-1 mRNA expression ranged between 21 and 39%, but there was no significant temporal pattern from 6 to 72 h. Similar to the induced expression of PAI-1 mRNA, cyclic strain also stimulated PAI-1 antigen secretion into the conditioned medium of HAECs at all time-points compared to static control cells (ANOVA treatment: $p<0.001$). Relative strain-induced increases in PAI-1 secretion were in the order of 16 to 47%, and the release rates of PAI-1 were significantly increased from 24–72 h ($p<0.01$ throughout).

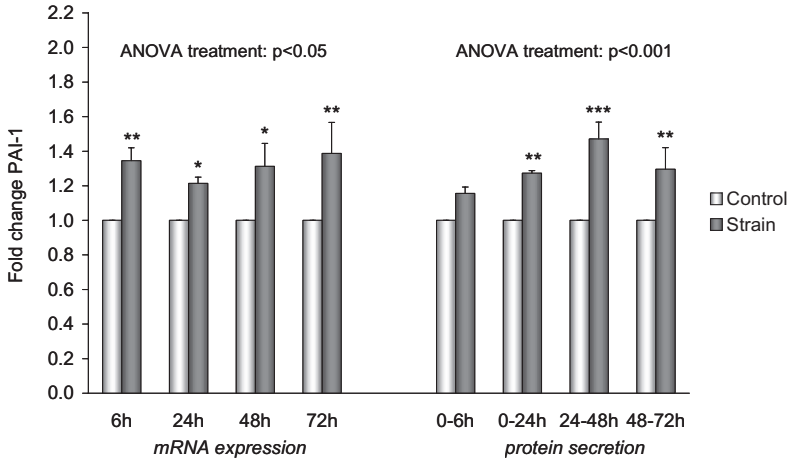

Figure 8. Cyclic strain induces PAI-1 mRNA expression and protein secretion. HAECs were exposed to 10% cyclic strain for 6-72 h and analyzed by real-time RT-PCR and ELISA for alterations in PAI-1 gene and protein expression, respectively. The results are expressed as fold change compared to static control cells. n=4, *p<0.05, **p<0.01 and ***p<0.001.

Study III

Laminar shear stress suppresses the t-PA gene

Cultured endothelial cells were exposed to steady laminar shear stress of either low (1.5 dyn/cm^2) or high (25 dyn/cm^2) magnitude in order to evaluate the effect of this biomechanical force on regulation of the t-PA gene. Shear stress was observed to time and magnitude dependently suppress t-PA gene expression (Figure 9A). Low shear stress stimulation of HUVECs resulted in a small and insignificant reduction of t-PA mRNA, while a distinct suppression was observed with high shear stress. In comparison with static control cells, high shear stress stimulation for 6 and 24 h reduced t-PA transcript levels by 20% ($p<0.05$) and 55% ($p<0.001$), respectively. When compared

to low sheared cells, the corresponding reductions where 13% ($p<0.05$) and 46% ($p<0.01$). To exclude the possibility that the response was restricted to endothelial vascular cells of umbilical origin or was an effect of the specific experimental setup, similar experiments were performed on HAECs and in a physiological pressurized vessel-like perfusion system developed at our laboratory [136]. These experiments confirmed the findings and showed an almost identical and magnitude dependent suppression of t-PA transcript with prolonged shear stress stimulation.

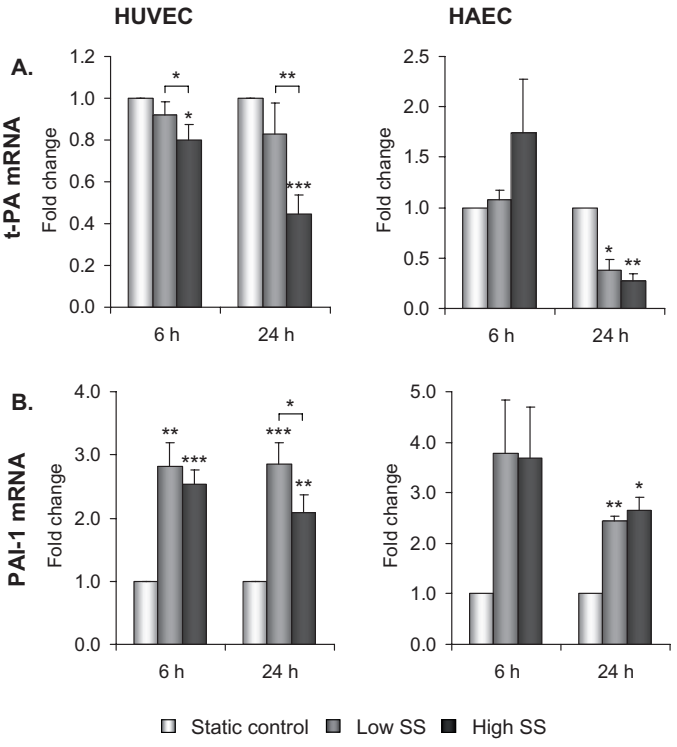

Figure 9. Laminar shear stress suppresses fibrinolytic gene expression. HUVECs (n=8) and HAECs (n=3) were exposed to low (1.5 dyn/cm^2) or high (25 dyn/cm^2) steady laminar shear stress for 6 or 24 h and analyzed by real-time RT-PCR for alterations in **A.** t-PA and **B.** PAI-1 gene expression. The results are expressed as fold change compared to static control cells. Unless indicated in the figure, statistical comparisons are made relative to static controls. *p<0.05, **p<0.01 and ***p<0.001.

Laminar shear stress induces the PAI-1 gene

Influence of shear stress on gene expression of PAI-1 was also investigated as previous documentation has been conflicting, reporting both stimulatory [137] and suppressing [112] effects. Both low and high shear stress induced PAI-1 mRNA by approximately 2-4 fold compared to static controls (Figure 9B). The induction was present in both

cell-types and at both studied time-points. While the shear-induced induction of PAI-1 in HAECs was observed to be independent of dose, high compared to low sheared HUVECs showed less distinct PAI-1 inductions ($p<0.05$ at 24 h).

Shear stress activates NF-kappaB and MAPK signaling

As the MAPK- and NF-κB cascades have previously been outlined as important pathways whereby signals originating from mechanical forces can lead to gene expression and protein synthesis [92], Western blotting experiments directed against the activated and phosphorylated forms of p65, ERK1/2, JNK, and p38 MAPK were performed. A rapid activation of all four signaling pathways was observed by either low or high shear stress stimulation (Figure 10). However, the pattern of activation was different with prolonged shear stress stimulation. NF-κB signaling, measured as phosphorylation of the p65 subunit, was gradually suppressed by increasing shear stress at 24 h. Further, while the initial increase in ERK1/2 activity was observed to level-off to basal levels by prolonged high shear stimulation, the elevated activity in low shear stressed cells was preserved. A similar pattern was observed for JNK signaling, where a relatively higher activity was observed in low sheared cells after 24 h of stimulation. On the other hand, the pattern for p38 MAPK signaling was the quite opposite. Early low shear stress induced activation of p38 MAPK was gradually decreased by prolonged stimulation, while high shear stressed cells was observed to have a sustained high p38 MAPK activity.

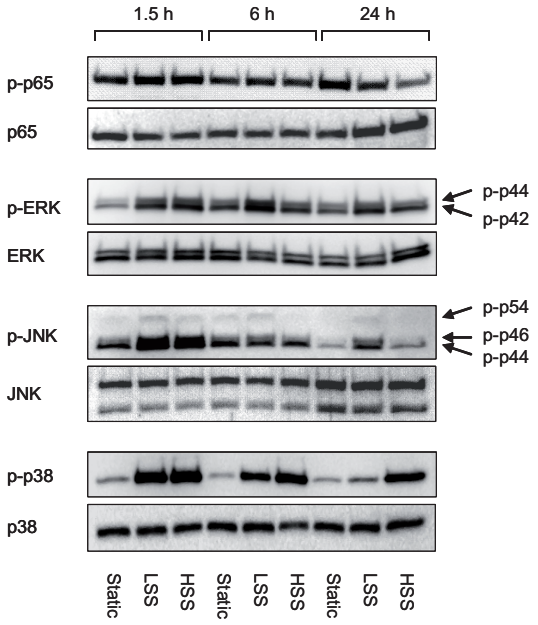

Figure 10. Shear stress activates NF-κB and MAPK signaling. HUVECs were exposed to low (LSS, 1.5 dyn/cm^2) or high (HSS, 25 dyn/cm^2) steady laminar shear stress for 1.5-24 h and analyzed by Western blotting for phosporylation of p65 (NFκB), ERK1/2, JNK and p38 MAPK. An unspecific signal is detected by the p-JNK antibody. As this signal is blocked by pretreatment of cells with the ERK1/2 specific inhibitor PD98059 (10 μM) it may represent p-p44. Data are representative of four independent experiments.

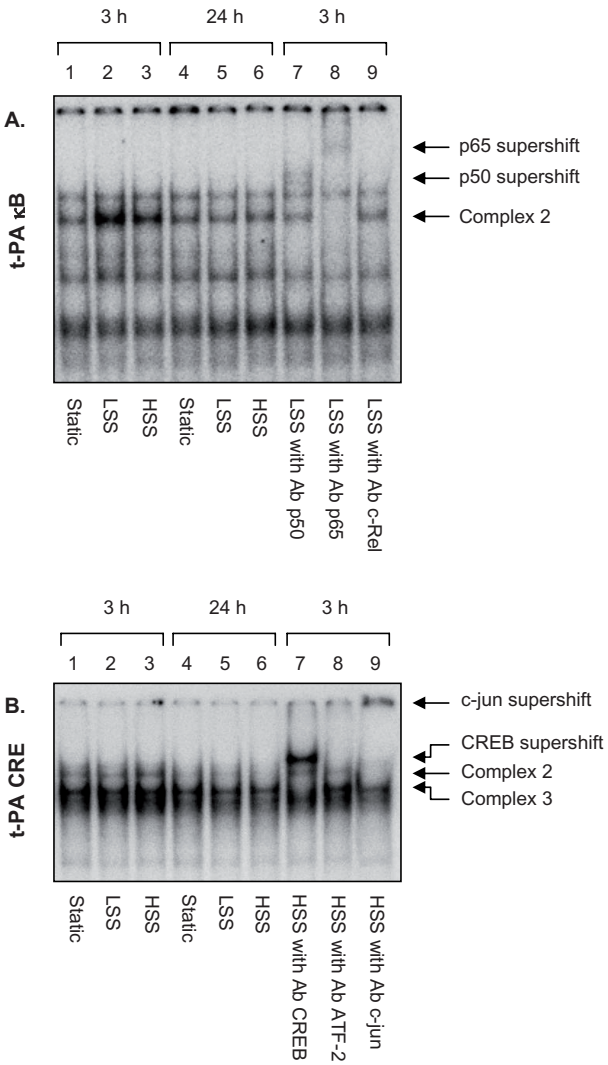

Figure 11. Shear stress enhances interactions with the t-PA κB and CRE elements. HUVECs were exposed to low (LSS, 1.5 dyn/cm^2) or high (HSS, 25 dyn/cm^2) steady laminar shear stress for 3 or 24 h and analyzed by EMSA for nuclear protein interactions with t-PA promoter specific elements. **A.** EMSA showing interactions with the t-PA κB probe. Lane 7-9 with addition of antibodies (Ab) directed against NF-κB subunits p50, p65 and c-Rel. Arrows point out the shear induced complex 2 as well as supershifts with p50 and p65 antibodies. **B.** EMSA showing interactions with the t-PA CRE probe. Lane 7-9 with addition of antibodies directed against CRE binding proteins CREB, ATF-2 and c-jun. Arrows point out the shear induced complex 2 and 3 as well as supershifts with c-jun and CREB antibodies. Data in each panel are representative of seven independent experiments.

Shear stress enhances interactions with the t-PA kappaB and CRE elements

EMSA experiments directed against five putative t-PA promoter specific sites were performed in order to determine the possible involvement of these sites in the shear stress induced suppression of t-PA. Short-term shear stimulation enhanced nuclear protein interactions with the t-PA specific κB and CRE probes, but no alterations in binding were observed after prolonged shear stimulation (Figure 11). Although the response was observed with both low and high shear, the magnitude of shear was observed to affect binding to the two sites in different ways. The increment in κB binding was consistently more profound in low shear stress stimulated samples, while high sheared cells showed the highest CRE binding. Supershift experiments identified an enhanced binding of p50/p65 heterodimers (NF-κB) to the κB site and an enhanced binding of the transcription factors CREB and c-jun to the CRE site. Nuclear protein binding to the SSRE, GC box II and GC box III elements in the t-PA promoter was found unaffected by shear stress stimulation.

Study IV

TNF-alpha suppresses t-PA

Effects of the proinflammatory cytokine TNF-α on t-PA gene regulation were evaluated. Prolonged stimulation (24 or 48 h) of HUVECs with moderate doses of recombinant TNF-α resulted in a distinct reduction of t-PA gene expression, while a less pronounced reduction was observed after 6 h (Figure 12A). The reduction attained statistical significance ($p<0.01$) at the two later time-points and with all three studied doses of TNF-α (0.1, 1.0 and 10 ng/ml). The two higher doses resulted in 65% suppression of t-PA gene expression compared to untreated control cells. The relevance of these gene regulatory effects was recently confirmed in experiments determining effects of TNF-α on t-PA release. Stimulation with 1 or 10 ng/ml TNF-α resulted in a time dependent reduction of t-PA, which during the time-period 24-48 h attained statistical significance (40% reduction, $p<0.01$) (Figure 12B).

TNF-alpha mediated suppression of t-PA is NF-kappaB and p38 MAPK dependent

To investigate the underlying mechanism of cytokine induced t-PA gene suppression, HUVECs were preincubated with selective inhibitors of the main signaling pathways activated by TNF-α. These studies showed that the response is preferentially an effect of NF-κB activation, but also pointed out the importance of p38 MAPK signaling (Figure 13). Inhibition of NF-κB (6 μM parthenolide) or p38 MAPK (25 μM SB203580) reduced the suppressive effect of TNF-α from 48% to 20 and 27%, respectively ($p<0.05$ for both). Due to the lack of a complete inhibition, experiments were performed in which cells were treated with a combination of the two inhibitors. Simultanous inhibition of NF-κB and p38 MAPK resulted in a striking counteraction of the TNF-α response, where the reduction of t-PA mRNA was completely abolished and shifted from a reduction to an induction. TNF-α stimulation of parthenolide and SB203580 treated cells resulted in a 39% induction, in comparison to a 48% reduction in the absence of inhibitors ($p<0.001$).

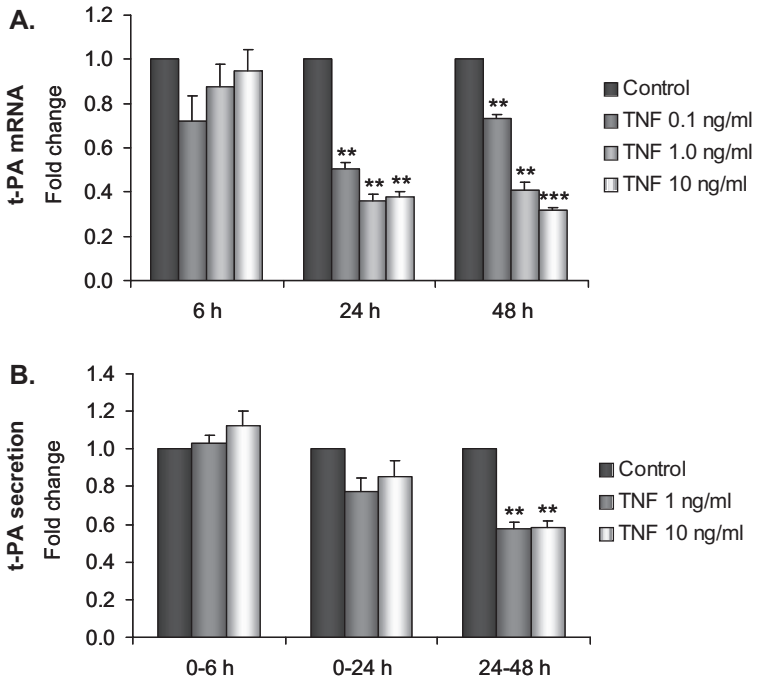

Figure 12. Prolonged TNF-α stimulation suppresses t-PA mRNA expression and protein secretion. HUVECs were exposed to 0.1-10 ng/ml TNF-α for 6-48 h and analyzed by real-time RT-PCR and ELISA for alterations in **A.** t-PA gene expression and **B.** t-PA protein secretion, respectively. n=3. **p<0.01 and ***p<0.001 compared to untreated control cells.

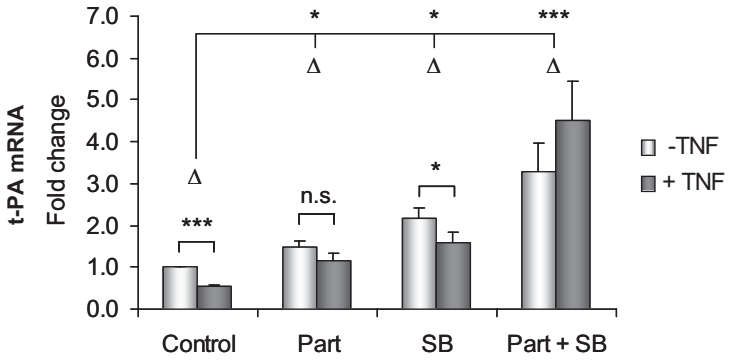

Figure 13. TNF-α mediated suppression of t-PA is NF-κB and p38 MAPK dependent. HUVECs were preincubated with synthetic inhibitors directed against NF-κB (6 μM parthenolide) and/or p38 MAPK (25 μM SB203580) for 1 h and thereafter stimulated with 1 ng/ml TNF-α for 24 h. Alterations in t-PA gene expression were analyzed by real-time RT-PCR and relevant statistical comparisons are pointed out in the figure. Δ indicating relative difference between cells stimulated with TNF-α and respective non-TNF-α stimulated control. n=6, *p<0.05 and ***p<0.001.

NF-kappaB, p38 MAPK and JNK signaling are involved in basal t-PA expression

Incubation of cells with each of the used inhibitors showed involvement of all three studied signaling pathways in basal t-PA gene expression. Inhibition of NF-κB or p38 MAPK induced t-PA mRNA expression by approximately 50% ($p<0.05$) and 120% ($p<0.01$), respectively (Figure 13). t-PA transcript was on the other hand reduced by 60% when JNK was inhibited with 10 μM SP600125 ($p<0.01$). These data indicated presence of two suppressive signaling pathways (NF-κB and p38 MAPK) and one inductive (JNK) for the t-PA gene expression in HUVECs.

TNF-alpha enhances interactions with the t-PA kappaB and CRE elements

EMSA experiments directed against either a consensus NF-κB or the t-PA specific κB element showed increased binding of nuclear proteins from TNF-α stimulated cells (Figure 14A). The binding patterns were almost identical for the two sites and showed strongly induced binding at both 3 and 24 h. Supershift experiments identified the cytokine induced shift (complex 2) to represent binding of the NF-κB subunits p50 and p65. TNF-α was observed to increase binding also to a consensus AP-1 and the t-PA specific, and AP-1 like, CRE element (Figure 14B). The similarity in binding patterns was striking between these two sites and showed strong and moderate inductions following 3 and 24 h of stimulation, respectively. These experiments indicated activation of AP-1 by TNF-α and an enhanced binding of proteins from the AP-1 family to the t-PA CRE site. However, supershift experiments identified the TNF-α induced t-PA CRE shift (complex 3) to represent binding of CREB.

Both parthenolide and SB203580 was observed to exert inhibitory effects on the TNF-α induced shifts. Inhibition of NF-κB with parthenolide reduced the intensity of the p50/p65-complex, while inhibition of p38 MAPK with SB203580 had no effect when used alone, but attenuated the p50/p65-complex when used in combination with parthenolide. This indicated that p38 MAPK inhibition augments the inhibitory effect of parthenolide on NF-κB activation by TNF-α. Furthermore, the increased binding to the t-PA CRE by TNF-α stimulation was observed to be counteracted by p38 MAPK inhibition, most notably in samples stimulated for 24 h.

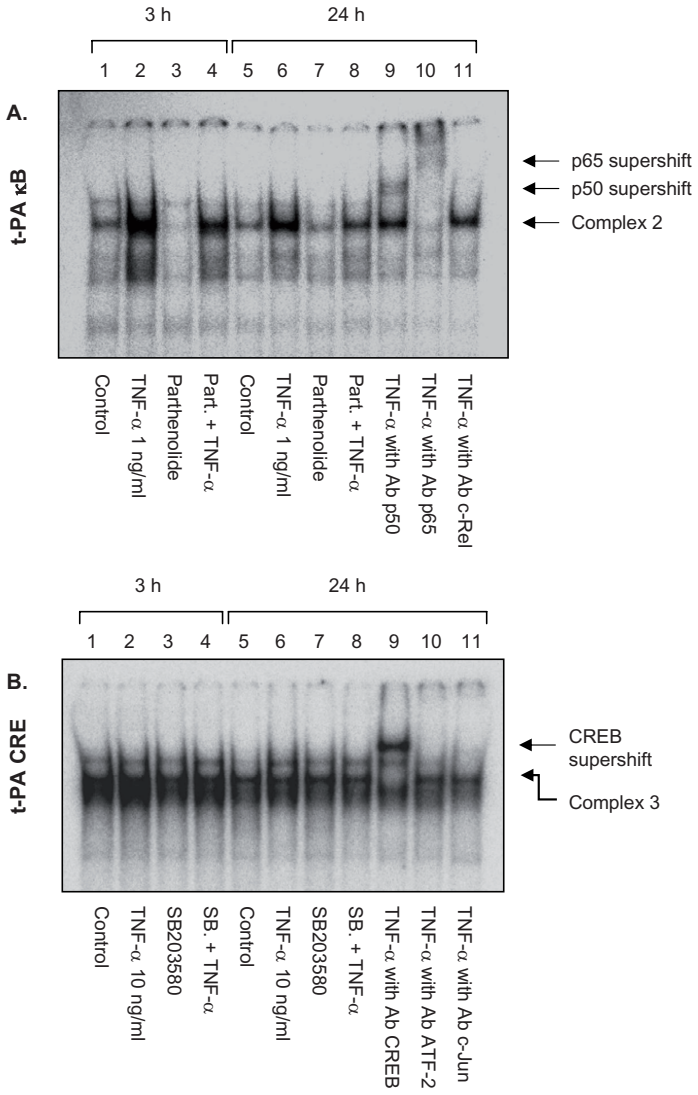

Figure 14. TNF-α enhances interactions with the t-PA κB and CRE elements. **A.** HUVECs were preincubated with the NF-κB inhibitor parthenolide (6 μM) for 1 h and thereafter stimulated with 1 ng/ml TNF-α for 3 and 24 h. EMSA was used to detect nuclear protein interactions with the t-PA specific κB probe. Lane 9-11 with the addition of antibodies (Ab) directed against NF-κB subunits p50, p65 and c-Rel. Arrows indicate supershifts with p50 and p65 antibodies as well as the TNF-α induced complex 2. **B.** HUVECs were preincubated with the p38 MAPK inhibitor SB203580 (25 μM) for 1 h and thereafter stimulated with 10 ng/ml TNF-α for 3 and 24 h. EMSA was used to detect nuclear protein interactions with the t-PA promoter specific CRE probe. Lane 9-11 with the addition of antibodies directed against CREB, ATF-2 and c-jun. Arrows indicate supershift with CREB antibody as well as the TNF-α induced complex 3. Data in each panel are representative of three independent experiments.

DISCUSSION

Previous work by our group has shown that both patients with primary and secondary hypertension have a reduced capacity to release t-PA upon stimulation [4, 5], a defect that is likely to contribute to the enhanced risk for arterial occlusion and tissue infarction in these subjects. The mechanism of this impairment is unclear although experimental studies have indicated that it could be a direct effect of the elevated blood pressure [3]. The present studies have focused on further exploring the underlying mechanisms of this impaired t-PA response.

Antihypertensive treatment restores impaired t-PA response

In order to investigate if the impairment could be restored by lowering the blood pressure, hypertensive subjects were assessed for stimulated t-PA release before and after antihypertensive treatment. The findings show for the first time that the capacity for stimulated t-PA release can be significantly improved by blood pressure lowering. Not only did treatment increase the amount of t-PA released, but it also improved the temporal pattern of the response with an earlier onset and a higher peak release rate. In all 6 of the patients with a delayed t-PA release response, the time to peak was improved. The changes were of similar magnitude in the lisinopril and felodipine groups, suggesting that the improvement was related to the blood pressure effect *per se*.

Our previous *in vivo* studies in humans and in experimental porcine models have shown that the acute t-PA response is rapid and powerful under physiological conditions [47, 48, 138, 139]. Indeed, a prompt onset of the response with a short delay until the peak of the secretory response is probably pivotal for its antithrombotic effect, because t-PA is approximately twice as effective when t-PA is present during clot formation than in dissolving an existing thrombus [19, 20]. In healthy humans, acute release of t-PA can be initiated within <1 minute, and the release rate can increase manifold with stimulation [47, 58, 139, 140]. If the response is slow and defective, as it appears to be in patients with untreated hypertension, it may reduce the potential for a timely activation of the fibrinolysis in case of an atherothrombotic event.

Several observations suggest that lowering blood pressure reduces the risk of atherothrombosis [141]. Restoration of the capacity for an effective fibrinolytic response could be one of the looked-for mechanisms behind this effect. Despite its potential implications, however, the effects of antihypertensive therapy on the capacity for activation of the local fibrinolytic system in hypertension have not been defined. A few previous studies have investigated the effect of blood pressure-lowering drugs on the plasma levels of fibrinolytic factors. In contrast to a calcium antagonist and an angiotensin II type 1 receptor blocker, 2 ACE inhibitors (enalapril and quinapril) improved plasma fibrinolysis [142, 143]. These findings suggest that ACE inhibition has a more favorable effect on t-PA production. Unfortunately, local endothelial t-PA secretion rates cannot be inferred from these studies, because of the following: (1) mixed venous plasma t-PA represents the integrated release from the whole vasculature, and (2) the rapid degradation of t-PA by the liver makes steady-state levels very sensitive

even to minute changes in hepatic clearance [144]. Furthermore, as shown by us in a previous study [2], baseline plasma levels do not predict the individual's capacity for activation of the acute release response, which is likely to be the key determinant of a successful clot resolution.

More specifically investigating the effect of ACE inhibition on the capacity for stimulated local t-PA release, Minai *et al*. [140] and Matsumoto *et al*. [145] studied the response to intracoronary infusion of bradykinin in patients with atypical chest pain and hypertension, respectively. In both studies, ACE inhibition was found to potentiate bradykinin-induced t-PA release, and a similar effect was also reported in a forearm study [146, 147]. However, these observations do not necessarily indicate that there is a specific improvement in endothelial t-PA synthesis or release capacity, because it cannot be ruled out that the effect is because of an increased bioavailability of bradykinin when its degradation is blocked by inhibition of ACE. Indeed, a recent study by Pretorius *et al*. [148] confirmed that the effect of ACE inhibition on t-PA release was bradykinin dependent, and the effect could be abolished by the bradykinin B_2-receptor blocker HOE 140. To avoid a confounding effect of this potential pharmacological interaction, we used substance P in the present study to ensure that t-PA release was induced through a bradykinin-independent pathway. Thus, our findings clearly suggest a cellular mechanism to be responsible for the improved t-PA secretory response by antihypertensive therapy in hypertensive patients.

Taken together with our previous observations, it is likely that the impaired capacity for t-PA release in hypertensive patients reflects a diminished protein synthesis with depletion of the intracellular storage pools from which it is released on stimulation [41]. The fact that an improved fibrinolytic capacity was observed with both drugs supports the concept that the effect is primarily related to the blood pressure decrease as such. However, the observation that the improvement was somewhat greater with the ACE inhibitor may indicate that there are additional, drug-specific effects. This important issue needs to be addressed in future studies.

In contrast to the improvement in t-PA release, we found no effect of blood pressure treatment on the vasodilatory capacity of the endothelium. Thus, the regulation of endothelial fibrinolysis and vasodilation appears, at least to some extent, to be distinct phenomena. Previous studies on the effect of chronic blood pressure treatment have shown either no change [149, 150] or improvement [151-154] in the vasomotor functions. The positive findings have been most consistent for treatment with ACE inhibitors and calcium channel blockers and when bradykinin or acetylcholine/methacholine have been used as stimuli. To our knowledge, the effects of blood pressure lowering on substance P-induced vasodilation have not been described previously.

In conclusion, the impaired capacity for acute release of t-PA from the vascular endothelium in patients with primary hypertension can be restored by antihypertensive therapy. Because a rapid and powerful release of t-PA is essential for prevention of thrombus formation in case of intravascular clot formation, this observation provides a novel mechanism behind the preventive effect of blood pressure-lowering therapy on the risk for atherothrombotic events.

Prolonged cyclic strain impairs fibrinolysis

Next, we wanted to explore the mechanistic link between high blood pressure and low t-PA expression. Because increased mechanical tension acting on the vessel wall is a component of elevated blood pressure, we hypothesized that an elevated tensile stress could be the mechanism behind the impaired t-PA response. The impact of this particular force was therefore studied in cultured vascular endothelial cells. As hypothesized, prolonged cyclic strain (≥48 h) was shown to decrease endothelial fibrinolytic capacity by a suppression of both t-PA gene and protein expression. Interestingly, cyclic strain was also observed to induce PAI-1 transcript and protein secretion, thereby illustrating a dual suppressive effect on the fibrinolytic system. The PAI-1 effect is in good agreement with existing litterature [107, 108, 155]. However, our study is the first to document that the inductive response is present also on the level of gene expression. Further, the stimulatory effect of strain on PAI-1 gene expression and secretion of PAI-1 may contribute to the elevated circulating plasma PAI-1 frequently observed in hypertensive patients [156].

Regarding t-PA, a few studies have previously reported on the effects of strain. These have either reported unaltered [107, 108] or induced [106] secretion of t-PA. The inconsistency in observed strain effects is likely to be explained by differences in the experimental setups; *e.g*. the mode, magnitude and duration of the strain stimulation, the used assay techniques, or by the choice of studied cell type. Our results differ the most from those presented by Iba and Sumpio, who reported an elevated secretion of t-PA in cells strained for 1-5 days [106], an induction of t-PA mRNA after 24 h of cyclic strain [109], and a rapid and transient increase in t-PA promoter activity in transfected cells stimulated with cyclic strain for up to 48 h [157]. A major difference between these studies and our is that the former used a previous version of the Flexcell strain device, which excludes a fixed post, and as a result of this the entire membrane is flexed, consequently exposing the attached cells to a much less defined strain. Furthermore, our data showed an early inductive effect of strain on t-PA mRNA and protein expression, which by time declined and shifted to a suppression. It is therefore possible that at least some of the inductive responses presented by Sumpios group are masked by this biphasic temporal regulation of t-PA.

The presented data indicate that an increased tensile stress, imposed on the endothelium *in vivo* by elevated blood pressure, could be a possible explanation for the impaired t-PA release observed in patients with hypertension. However, the observed strain effects were relatively modest indicating involvement also of other factors. This led us to speculate that the other major haemodynamic force component, frictional shear stress, could be involved in the impaired t-PA response. This is conceivable since vascular remodeling and arterial stiffness often follow progression of the hypertensive state, thereby resulting in exposure of the vascular endothelium to elevated shear stress.

Laminar shear stress impairs fibrinolytic gene expression

In order to examine if high shear stress down-regulates t-PA expression, perfusion experiments on cultured endothelial cells were set up. Interestingly, these experiments

showed a time and magnitude dependent suppression of t-PA gene expression with steady laminar shear stress stimulation. Furthermore, compared to strain, shear stress appeared to be a stronger determinant of fibrinolytic activity, since prolonged high (25 dyn/cm^2) shear was observed to result in an approximately twice as powerful repression of t-PA transcript.

In contrast to our observations, a few previous studies have indicated that shear stress induces t-PA gene expression in cultured endothelial cells [111-113], but the available reports date back to the early 90s when direct gene expression quantification methods were not available. Diamond and co-workers originally reported on a marked induction of t-PA mRNA by prolonged high shear stimulation (25 dyn/cm^2), but in a more recent study the same laboratory was unable to reproduce these data [158]. Further, Malek *et al*. reported on an early (up to 9 h) induction of t-PA expression in bovine aortic endothelial cells exposed to elevated shear stress (36 dyn/cm^2). As we also observed an early transient induction in aortic endothelial cells, and have previously reported on a moderate induction of t-PA transcript in short-term high sheared intact human conduit vessels [114], the data taken together suggest that also shear stress regulates t-PA in a biphasic temporal manner. However, we believe that the suppression after long-term shear/strain exposure is likely to more closely resemble the *in vivo* situation with chronic elevated biomechanical stress levels. As the shear effect is somewhat controversial, it is of note that we were able to reproduce this effect in endothelial cells of different vascular origin and in an entirely different experimental setup. The validity of the responses was also strenghtened by confirming previous documentation on shear-dependent eNOS and VCAM-1 regulation [159-161].

To elucidate the mechanism behind shear-dependent suppression of t-PA, gel shift experiments directed against five putative regulatory elements in the t-PA promoter were performed. These revealed enhanced binding of NF-κB and CREB to the κB and CRE sites, respectively. The induced κB binding was preferentially associated with short-term low shear stress stimulation, while short-term high shear stress induced the strongest CRE binding. However, neither of the two sites showed any altered binding after prolonged shear exposure, indicating that the repressed t-PA expression probably involves also other mechanisms. Furthermore, a direct link between the suppressed t-PA expression and the SSRE, GC box II or III could not be established, as we could not detect any altered binding to any of the three sites. Of course, involvement of these sites can not be excluded as shear stress has occasionally been reported to mediate gene regulatory effects by phosporylating transcription factors, like *e.g.* Sp1, without increasing its binding to promoter elements [104].

Western blotting on sheared samples showed activation of all studied signaling pathways with different temporal patterns. The most notable difference after prolonged stimulation was the sustained high p38 MAPK activity observed with high shear stress. As CREB is an effector molecule of p38 MAPK signaling this provides a potential link to the suppressed t-PA expression after prolonged high shear stimulation. Moreover, a high p38 MAPK activity is in line with several reports describing characteristics of high shear stressed cells. For example, cellular adaptive processes such as shear induced cell alignment and chromatin remodeling have been described to be p38 MAPK dependent [101, 162].

Thus, although laminar shear stress of physiologic levels has been shown to convey some atheroprotective effects on the vascular wall and retard atheroma formation [163], the present findings with suppressed t-PA expression, but also induced PAI-1 expression, indicate that such positive effects of shear do not necessarily extend to those exerted on the fibrinolytic system.

TNF-alpha suppresses t-PA expression by a NF-kappaB and p38 MAPK dependent mechanism

An inflammatory component is another potential contributor to the impaired t-PA response, as the hypertensive state is often associated with a low-grade inflammation [164-168]. To investigate this possiblity we exposed endothelial cultures to moderate doses of the prototypic proinflammatory cytokine TNF-α and observed a distinct suppression of t-PA gene expression with extended stimulation (≥24 h). Previous documentation on this cytokine and its effect on endothelial t-PA gene expression is limited to a small number of studies preferentially describing reductions of t-PA mRNA in response to TNF-α [69, 125, 169]. A single study has reported an induction [170], but a major limitation of that study is the short observation time (4 h).

It is likely that the TNF-α induced suppression of t-PA observed in our report is an effect of preferentially NF-κB activation, since we observed a decreased nuclear localization of NF-κB and an almost complete counteraction of the suppressed t-PA gene expression upon inhibition of the NF-κB signaling pathway. Furthermore, EMSA experiments indicated that NF-κB may exert its effects by interacting with the t-PA promoter specific κB element. This is somewhat surprising because NF-κB is traditionally considered to primarily mediate transcriptional up-regulation. However, there are studies showing that NF-κB under some conditions can function as a transcriptional repressor of haemostatic molecules in endothelial cells [171, 172]. One mechanism by which this could occur is by transcriptional interference caused by the direct binding of inhibitory NF-κB dimers to promoter κB sites. These inhibitory complexes usually consist of p50 or p52 homodimers lacking transcriptional activation domains [173]. This is a tempting explanation as in EMSA experiments we observed two supershifts with the p50 antibody, indicating presence of both translocated p50/p65 heterodimers and p50/p50 homodimers. An alternative mechanism by which NF-κB could reduce t-PA gene expression is by interfering with transcriptional co-activators (such as p300 and CBP) which are present in limited quantities. This mechanism has for example been implied in the NF-κB mediated negative regulation of the thrombomodulin gene [172]. Thirdly, it is possible that NF-κB could recruit transcriptional co-repressors (*e.g*. histone deacetylases) to the t-PA promoter [174].

On the other hand, the reduced t-PA suppression in experiments with p38 MAPK inhibition could be an effect of an inhibited activation of CREB, as inhibition of this signaling pathway was observed to reduce the TNF-α induced binding of CREB to t-PA CRE. In this context it is of interest that inhibition of p38 MAPK with SB203580 has previously been documented to block TNF-α induced CREB activation [175],

and that CREB binding to the t-PA CRE has been reported to mediate phorbol-12-myristate 13-acetate (PMA) induced down-regulation of t-PA i HT-1080 fibrosarcoma cells [71]. The p38 MAPK pathway has also been reported to increase binding of NF-κB to chromatin, at least partly by phosphorylation and phosphoacetylation of histone H3, leading to loosening of the nucleosome and an enhanced accessibility to NF-κB binding sites [176]. Although somewhat speculative, the abolished suppression of t-PA mRNA expression observed with combined inhibition of NF-κB and p38 MAPK signaling could therefore be an effect of a reduced translocation of NF-κB dimers to the nucleus together with a reduced access to NF-κB binding sites in the t-PA promoter.

Further, we show that NF-κB, p38 MAPK, and JNK signaling are all involved in regulation of basal t-PA gene expression. Our data suggest that activation of the NF-κB and p38 MAPK pathways reduces transcriptional activity of t-PA, while JNK activity acts by stimulating t-PA gene expression. This observation links directly to the suppressed t-PA expression with prolonged high shear stress stimulation, as the most profoundly activated signaling pathway was the elevated p38 MAPK activity.

As gene regulatory effects can not automatically be translated into alterations in protein synthesis, it is of note that we recently observed also t-PA secretion to be reduced upon stimulation with TNF-α. This effect is in accordance with previous *in vitro* studies, the majority of which describe a reduced t-PA secretion in response to TNF-α [125, 127, 169]. By contrast, *in vivo* studies on humans have shown substantial inductions of t-PA release when challenged with TNF-α [130, 131, 177, 178]. However, the experimental design and interpretation of obtained data in these studies are somewhat different. In the two reports from Newby's laboratory, the authors have used the perfused-forearm model to study acute effects of intraarterial TNF-α infusions in either healthy subjects [130] or in patients with stable coronary heart disease [177]. They conclude that TNF-α besides its stimulatory effect on t-PA release, also is associated with an impairment of endothelium-dependent vasomotor responses. Van Hinsbergh *et al*. [131] studied cancer patients that during 24 h received a continuous intravenous infusion of TNF-α. They reported elevated plasma concentrations of t-PA after 3 and 24 h of infusion, but speculate that this response may be secondary to a concomitant TNF-α induced generation of thrombin. Finally, van der Poll *et al*. [178] studied healthy subjects after an intravenous bolus injection of TNF-α and observed a rapid release of t-PA, which was followed by a subsequent inhibition of the fibrinolytic system.

Taken together, available data from different experimental set-ups indicate that TNF-α has an early stimulatory effect on t-PA, which shifts to a suppressing mode following prolonged stimulation. It is therefore possible that chronic elevated cytokine levels *in vivo*, by reducing t-PA synthesis and endothelial storage, may contribute to the impaired t-PA response in hypertension. In this context, it is interesting that chronic low-grade inflammation has been associated with a reduced capacity to release t-PA after venous occlusion of the forearm [179].

Towards a unifying hypothesis

The data presented in this thesis indicate that the impaired thromboprotective response in hypertensive patients is likely to be an effect of several contributing and interacting mechanisms. Our results suggest that the elevated blood pressure as such plays a pivotal role for the impairment, by imposing increased tensile stress on the vessel wall. However, the data also indicate that an enhanced shear stress could be a contributing factor, at least in subjects with a high-resistance type of hypertension. Elevated peripheral vascular resistance may in initial phases of the hypertensive process be an effect of neurogenic vasoconstriction. In later stages vascular resistance is typically maintained by the vascular remodeling processes that follow prolonged blood pressure elevation.

In addition to the haemodynamic effects, our results show that the low-grade inflammation that often accompanies hypertension also could be a factor that contributes to the reduced capacity for an efficient fibrinolytic response. Hypertension and inflammation could be related as a consequence of an underlying metabolic syndrome [180]. However, there is also substantial evidence to support a direct link between hypertension and inflammation, although it is not known whether inflammation is a cause or effect of the elevated blood pressure [181].

Several mechanisms that affect the pathophysiology of hypertension are known to be potentiated by proinflammatory agents. CRP, which is a powerful marker of increased risk for atherothrombotic events, has been shown to impair endothelial dependent vasorelaxation by suppressed production of NO and diminuation of its bioavailability [182]. Also, TNF-α has been shown to be associated with impaired vasodilation [130]. Moreover, vascular remodeling is stimulated by low-grade inflammation, which in turn is triggered by vasoconstrictive peptides such as angiotensin II and endothelin-1 [183]. Inflammation, and more specifically CRP, has further shown a significant relationship with increased arterial stiffness [184]. Interestingly, a recent report has shown that reduced NO availability is directly correlated to impaired capacity for t-PA release [185]. In addition, CRP has been shown to have a direct effect on t-PA. In cultured endothelial cells, CRP was observed to suppress t-PA expression, an effect that was mediated via generation of TNF-α and IL-1β [186].

All these mechanisms have the potential to amplify blood pressure and shear stress exposure of the endothelium and thereby contribute to the suppression of t-PA expression in the vascular endothelium. Furthermore, it is of note that the expression of the physiologic inhibitor of t-PA, PAI-1, is induced both by strain, shear stress, and proinflammatory cytokines. Thus, the data presented in this thesis suggest that enhanced haemodynamic and inflammatory stress may act in concert to put a double burden on the endothelial fibrinolytic system. In susceptible individuals, this may markedly reduce the chance of an effective and timely spontaneous thrombolysis of an evolving arterial thrombus. If so, this mechanism may help to explain the high risk of clinical atherothrombotic events in subjects with hypertension and inflammation.

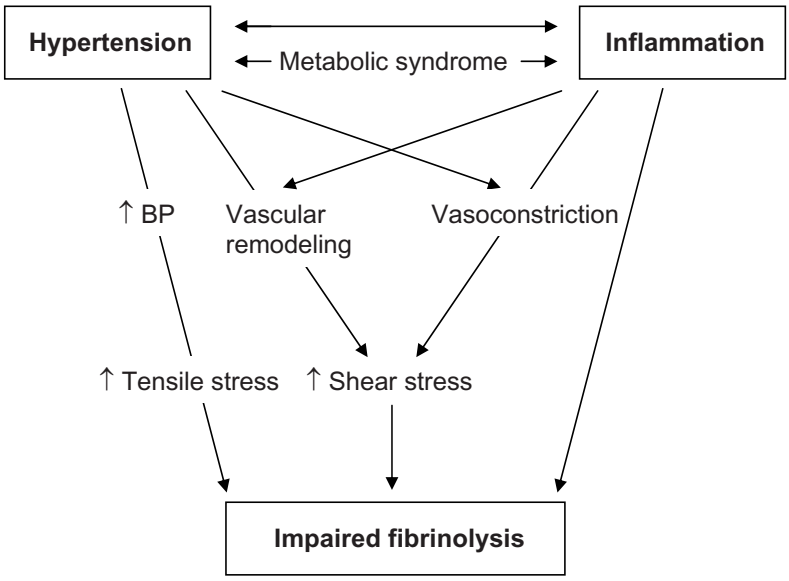

Figure 15. Shematic of potential mechanisms underlying an impaired fibrinolytic response.

CONCLUSIONS

The impaired capacity for stimulated t-PA release in hypertensive patients can be restored by antihypertensive therapy and this improvement seems to be related to the blood pressure reduction *per se* (Paper I).

Presented *in vitro* studies indicate that the defect t-PA response could be a consequence of an enhanced tensile stress (Paper II), shear stress (Paper III) and inflammatory stress (Paper IV) of the endothelium.

Prolonged cyclic strain impairs the endothelial fibrinolytic capacity by a dual action, with suppression of t-PA and induction of PAI-1 expression (Paper II).

Laminar shear stress magnitude dependently suppresses t-PA gene expression while shear stress as such induces PAI-1 transcript (Paper III).

The proinflammatory cytokine TNF-α suppresses endothelial t-PA expression by a NF-κB and p38 MAPK dependent mechanism. (Paper IV)

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hjärt- och hjärninfarkt uppstår vanligen till följd av att en blodpropp bildas i något av de kärl som försörjer dessa livsviktiga organ. Härigenom förhindras blodflödet helt eller delvis, ofta med allvarliga konsekvenser. Blodkärlets vägg har ett eget inneboende försvarssystem mot blodproppsbildning. Då en propp bildas aktiveras detta system, vilket bland annat innebär att ett blodproppsupplösande ämne frisätts till blodbanan. Detta kallas för vävnads-plasminogenaktivator (t-PA). Det är viktigt att ha stora depåer av t-PA i kärlväggen eftersom det minskar risken för att blodproppar skall byggas på och stoppa blodflödet i kärlet. I blodet finns även ämnen som kan blockera t-PAs propplösande förmåga. Det viktigaste av dessa är plasminogenaktivatorinhibitor-1 (PAI-1). t-PA bildas i huvudsak av endotelceller som utgör det innersta lagret i ett blodkärl. I endotelet upplagras stora mängder t-PA som vid behov snabbt kan frisättas till blodet. Vår forskningsgrupp har tidigare visat att personer med högt blodtryck har en försämrad förmåga att frisätta t-PA, något som troligen bidrar till att denna grupp löper ökad risk för att insjukna i hjärt- och hjärninfarkt.

I denna avhandling undersöktes eventuella bakomliggande orsaker till den defekta t-PA-responsen vid högt blodtryck. Arbetet har utförts på flera olika nivåer, dels genom att studera t-PA-frisättning hos patienter med högt blodtryck, dels genom att undersöka t-PA-frisättning i odlade endotelceller som stimulerats med faktorer som kan vara associerade med högt blodtryck, men även genom att studera syntesen av t-PA-proteinet i odlat endotel. Det sistnämnda har syftat till att öka förståelsen kring de mekanismer som bidrar till en minskad t-PA-produktion och därmed en tänkbar minskad kapacitet för frisättning.

I delarbete I ville vi undersöka huruvida den försämrade förmågan att frisätta t-PA går att förbättra genom att med blodtrycksbehandling sänka det förhöjda blodtrycket. I studien inkluderades patienter med högt blodtryck, och t-PA-frisättningsförmågan hos dessa undersöktes med hjälp av den s.k. perfunderade underarmsmodellen. Denna modell är utvecklad på vårt laboratorium och möjliggör studier av stimulerad t-PA-frisättning över ett isolerat organ, i detta fall underarmen. Patienterna undersöktes vid två tillfällen, dels innan och dels efter blodtrycksbehandling. Resultaten visade på kraftigt förbättrad förmåga att frisätta t-PA efter behandling. Behandlingen resulterade i både ökad mängd frisatt t-PA samt en snabbare frisättning. Då behandling med två olika blodtryckssänkande preparat gav likartade effekter, indikerade detta att den förbättrade t-PA-frisättningen var en direkt följd av det sänkta blodtrycket.

I delarbete II ville vi undersöka mekanismen bakom den defekta t-PA-frisättningen. Vi spekulerade kring om den skulle kunna bero på en ökad biomekanisk belastning av kärlväggen och därmed av endotelet. En direkt följd av ett förhöjt blodtryck är en ökad tensionskraft som sträcker ut kärlväggen. Vi undersökte med hjälp av ett kommersiellt biomekaniskt modellsystem effekten av denna isolerade kraft på odlade endotelceller. Studien visade minskat genuttryck (syntes) och minskad frisättning av t-PA vid långvarig cyklisk sträckstimulering. Intressant nog visade sig regleringen och frisättningen av PAI-1 gå åt motsatt håll. Dessa fynd indikerar att tensil belastning av kärlets endotel kan bidra till en försämrad förmåga att lösa upp blodproppar.

I delarbete III ville vi undersöka effekten av den andra huvudsakliga biomekaniska kraften shear stress. Shear stress är den friktionskraft som blodflödet utövar på kärlväggen och framför allt endotelet. Även ökad shear stress är en tänkbar effekt av långvarigt förhöjt blodtryck, då detta tillstånd i många fall resulterar i ökad kärlstyvhet och kärlombildning. Effekten av denna kraft undersöktes på liknande sätt i ett biomekaniskt modellsystem designat för flödesstimulering av odlade endotelceller. Studien visade en magnitudberoende minskning av genuttrycket för t-PA vid stimulering med shear stress. I likhet med tensil stimulering inducerade även shear stress syntesen av PAI-1, men denna var inte lika tydligt kopplad till magnituden av shear stress.

I delarbete IV utvärderades effekten av den inflammatoriska molekylen TNF-α på produktionen och frisättningen av t-PA i odlade endotelceller. Detta gjordes mot bakgrunden att en låggradig inflammation med förhöjda cirkulerande nivåer av inflammatoriska cytokiner, såsom TNF-α, ofta sammanfaller med risktillstånd för hjärt- och kärlsjukdom. Högt blodtryck är ett av dessa risktillstånd. Denna studie visade både minskat genuttryck och minskad frisättning av t-PA vid långvarig stimulering med moderata doser av TNF-α. Vidare kartlade vi de intracellulära signaleringsvägar genom vilka TNF-α reducerar t-PA-produktionen. Detta gjordes genom att med hjälp av inhibitoriska substanser hämma kända signaleringsvägar som aktiveras av TNF-α. Försöken visade att reduktionen av t-PA vid stimulering med TNF-α är en följd av aktivering av signaleringsvägarna NF-κB och p38 MAPK.

Sammanfattningsvis visar resultaten av denna avhandling att den försämrade kapaciteten för frisättning av det blodproppsupplösande ämnet t-PA vid högt blodtryck går att motverka genom att aktivt sänka blodtrycket. Vidare visar de experimentella studierna att de bakomliggande orsakerna till den defekta t-PA-responsen kan vara en hög biomekanisk belastning av kärlväggen, i form av tensil stress och shear stress, samt att ett inflammatoriskt inslag ytterligare kan försämra proppupplösningsförmågan.

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REFERENCES

1. Murray CJ, Lopez AD: Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. Lancet 1997, 349(9064):1498-1504.

2. Hrafnkelsdottir T, Gudnason T, Wall U, Jern C, Jern S: Regulation of local availability of active tissue-type plasminogen activator in vivo in man. J Thromb Haemost 2004, 2(11):1960-1968.

3. Sjogren LS, Doroudi R, Gan L, Jungersten L, Hrafnkelsdottir T, Jern S: Elevated intraluminal pressure inhibits vascular tissue plasminogen activator secretion and downregulates its gene expression. Hypertension 2000, 35(4):1002-1008.

4. Hrafnkelsdottir T, Wall U, Jern C, Jern S: Impaired capacity for endogenous fibrinolysis in essential hypertension. Lancet 1998, 352(9140):1597-1598.

5. Hrafnkelsdottir T, Ottosson P, Gudnason T, Samuelsson O, Jern S: Impaired endothelial release of tissue-type plasminogen activator in patients with chronic kidney disease and hypertension. Hypertension 2004, 44(3):300-304.

6. Reinhart WH: Shear-dependence of endothelial functions. Experientia 1994, 50(2):87-93.

7. Jaffe EA: Cell biology of endothelial cells. Hum Pathol 1987, 18(3):234-239.

8. Pries AR, Secomb TW, Gaehtgens P: The endothelial surface layer. Pflugers Arch 2000, 440(5):653-666.

9. Emeis JJ: Regulation of the acute release of tissue-type plasminogen activator from the endothelium by coagulation activation products. Ann N Y Acad Sci 1992, 667:249-258.

10. van den Eijnden-Schrauwen Y, Kooistra T, de Vries RE, Emeis JJ: Studies on the acute release of tissue-type plasminogen activator from human endothelial cells in vitro and in rats in vivo: evidence for a dynamic storage pool. Blood 1995, 85(12):3510-3517.

11. Lupu C, Kruithof EK, Kakkar VV, Lupu F: Acute release of tissue factor pathway inhibitor after in vivo thrombin generation in baboons. Thromb Haemost 1999, 82(6):1652-1658.

12. Buga GM, Gold ME, Fukuto JM, Ignarro LJ: Shear stress-induced release of nitric oxide from endothelial cells grown on beads. Hypertension 1991, 17(2):187-193.

13. Frangos JA, Eskin SG, McIntire LV, Ives CL: Flow effects on prostacyclin production by cultured human endothelial cells. Science 1985, 227(4693):1477-1479.

14. del Zoppo GJ: Thrombolysis in cerebrovascular ischemia. In: PG-G, editor Fibrinolysis in disease, Pt 2: Boca Raton 1995:156-164.

15. Rentrop KP, Feit F, Sherman W, Thornton JC: Serial angiographic assessment of coronary artery obstruction and collateral flow in acute myocardial infarction. Report from the second Mount Sinai-New York University Reperfusion Trial. Circulation 1989, 80(5):1166-1175.

16. Lijnen HR, Collen D: Mechanisms of physiological fibrinolysis. Baillieres Clin Haematol 1995, 8(2):277-290.

17. Collen D: On the regulation and control of fibrinolysis. Edward Kowalski Memorial Lecture. Thromb Haemost 1980, 43(2):77-89.

18. Rijken DC, Collen D: Purification and characterization of the plasminogen activator secreted by human melanoma cells in culture. J Biol Chem 1981, 256(13):7035-7041.

19. Brommer EJ: The level of extrinsic plasminogen activator (t-PA) during clotting as a determinant of the rate of fibrinolysis; inefficiency of activators added afterwards. Thromb Res 1984, 34(2):109-115.

20. Fox KA, Robison AK, Knabb RM, Rosamond TL, Sobel BE, Bergmann SR: Prevention of coronary thrombosis with subthrombolytic doses of tissue-type plasminogen activator. Circulation 1985, 72(6):1346-1354.

21. Lijnen HR, Collen D: Endothelium in hemostasis and thrombosis. Prog Cardiovasc Dis 1997, 39(4):343-350.

22. Dano K, Andreasen PA, Grondahl-Hansen J, Kristensen P, Nielsen LS, Skriver L: Plasminogen activators, tissue degradation, and cancer. Adv Cancer Res 1985, 44:139-266.

23. Chmielewska J, Ranby M, Wiman B: Evidence for a rapid inhibitor to tissue plasminogen activator in plasma. Thromb Res 1983, 31:427-436.

24. Kruithof EK, Tran-Thang C, Ransijn A, Bachmann F: Demonstration of a fast-acting inhibitor of plasminogen activators in human plasma. Blood 1984, 64(4):907-913.

25. Verheijen JH, Chang GT, Kluft C: Evidence for the occurrence of a fast-acting inhibitor for tissue-type plasminogen activator in human plasma. Thromb Haemost 1984, 51(3):392-395.

26. Bennett B, Croll A, Ferguson K, Booth NA: Complexing of tissue plasminogen activator with PAI-1, alpha 2-macroglobulin, and C1-inhibitor: studies in patients with defibrination and a fibrinolytic state after electroshock or complicated labor. Blood 1990, 75(3):671-676.

27. Booth NA, Walker E, Maughan R, Bennett B: Plasminogen activator in normal subjects after exercise and venous occlusion: t-PA circulates as complexes with C1-inhibitor and PAI-1. Blood 1987, 69(6):1600-1604.

28. Rijken DC, Juhan-Vague I, Collen D: Complexes between tissue-type plasminogen activator and proteinase inhibitors in human plasma, identified with an immunoradiometric assay. J Lab Clin Med 1983, 101(2):285-294.

29. Hoylaerts M, Rijken DC, Lijnen HR, Collen D: Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. J Biol Chem 1982, 257(6):2912-2919.

30. Loscalzo J: Structural and kinetic comparison of recombinant human single- and two-chain tissue plasminogen activator. J Clin Invest 1988, 82(4):1391-1397.

31. Carmeliet P, Collen D: Gene targeting and gene transfer studies of the biological role of the plasminogen/plasmin system. Thromb Haemost 1995, 74(1):429-436.

32. Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J, Bronson R, De Vos R, van den Oord JJ, Collen D, Mulligan RC: Physiological consequences of loss of plasminogen activator gene function in mice. Nature 1994, 368(6470):419-424.

33. Matsuno H, Kozawa O, Niwa M, Ueshima S, Matsuo O, Collen D, Uematsu T: Differential role of components of the fibrinolytic system in the formation and removal of thrombus induced by endothelial injury. Thromb Haemost 1999, 81(4):601-604.

34. Waugh JM, Kattash M, Li J, Yuksel E, Kuo MD, Lussier M, Weinfeld AB, Saxena R, Rabinovsky ED, Thung S et al: Gene therapy to promote thromboresistance: local overexpression of tissue plasminogen activator to prevent arterial thrombosis in an in vivo rabbit model. Proc Natl Acad Sci U S A 1999, 96(3):1065-1070.

35. Kathiresan S, Yang Q, Larson MG, Camargo AL, Tofler GH, Hirschhorn JN, Gabriel SB, O'Donnell CJ: Common genetic variation in five thrombosis genes and relations to plasma hemostatic protein level and cardiovascular disease risk. Arterioscler Thromb Vasc Biol 2006, 26(6):1405-1412.

36. Rijken DC: Plasminogen activators and plasminogen activator inhibitors: biochemical aspects. Baillieres Clin Haematol 1995, 8(2):291-312.

37. Emeis JJ: Mechanisms involved in short-terms changes in blood levels of t-PA. In: Kluft C, editor Tissue-type plasminogen activator: Physiological and clinical aspects Boca Raton: CRC Press 1988:21-35.

38. Emeis JJ: Normal and abnormal endothelial release of tissue-type plasminogen activator. In: Glas-Greenwald P, editor Fibrinolysis in disease Boca Raton: CRC Press 1995:55-64.

39. Kooistra T, Schrauwen Y, Arts J, Emeis JJ: Regulation of endothelial cell t-PA synthesis and release. Int J Hematol 1994, 59(4):233-255.

40. Shatos MA, Doherty JM, Stump DC, Thompson EA, Collen D: Oxygen radicals generated during anoxia followed by reoxygenation reduce the synthesis of tissue-type plasminogen activator and plasminogen activator inhibitor-1 in human endothelial cell culture. J Biol Chem 1990, 265(33):20443-20448.

41. Emeis JJ, van den Eijnden-Schrauwen Y, van den Hoogen CM, de Priester W, Westmuckett A, Lupu F: An endothelial storage granule for tissue-type plasminogen activator. J Cell Biol 1997, 139(1):245-256.

42. Tranquille N, Emeis JJ: Protein synthesis inhibition by cycloheximide does not affect the acute release of tissue-type plasminogen activator. Thromb Haemost 1989, 61(3):442-447.

43. Jern C, Selin L, Jern S: Application of the perfused-forearm model to study release mechanisms of tissue-type plasminogen activator in man. Fibrinolysis 1994, 8:13-15.

44. Jern C, Selin L, Jern S: In vivo release of tissue-type plasminogen activator across the human forearm during mental stress. Thromb Haemost 1994, 72(2):285-291.

45. van den Eijnden-Schrauwen Y, Atsma DE, Lupu F, de Vries RE, Kooistra T, Emeis JJ: Involvement of calcium and G proteins in the acute release of tissue-type plasminogen activator and von Willebrand factor from cultured human endothelial cells. Arterioscler Thromb Vasc Biol 1997, 17(10):2177-2187.

46. Giles AR, Nesheim ME, Herring SW, Hoogendoorn H, Stump DC, Heldebrant CM: The fibrinolytic potential of the normal primate following the generation of thrombin in vivo. Thromb Haemost 1990, 63(3):476-481.

47. Hrafnkelsdottir T, Erlinge D, Jern S: Extracellular nucleotides ATP and UTP induce a marked acute release of tissue-type plasminogen activator in vivo in man. Thromb Haemost 2001, 85(5):875-881.

48. Bjorkman JA, Jern S, Jern C: Cardiac sympathetic nerve stimulation triggers coronary t-PA release. Arterioscler Thromb Vasc Biol 2003, 23(6):1091-1097.

49. Newby DE, Wright RA, Ludlam CA, Fox KA, Boon NA, Webb DJ: An in vivo model for the assessment of acute fibrinolytic capacity of the endothelium. Thromb Haemost 1997, 78(4):1242-1248.

50. Brown NJ, Gainer JV, Stein CM, Vaughan DE: Bradykinin stimulates tissue plasminogen activator release in human vasculature. Hypertension 1999, 33(6):1431-1435.

51. Wall U, Jern C, Jern S: High capacity for tissue-type plasminogen activator release from vascular endothelium in vivo. J Hypertens 1997, 15(12 Pt 2):1641-1647.

52. Lowe GD, Danesh J, Lewington S, Walker M, Lennon L, Thomson A, Rumley A, Whincup PH: Tissue plasminogen activator antigen and coronary heart disease. Prospective study and meta-analysis. Eur Heart J 2004, 25(3):252-259.

53. Ridker PM, Hennekens CH, Stampfer MJ, Manson JE, Vaughan DE: Prospective study of endogenous tissue plasminogen activator and risk of stroke. Lancet 1994, 343(8903):940-943.

54. Ridker PM, Vaughan DE, Stampfer MJ, Manson JE, Hennekens CH: Endogenous tissue-type plasminogen activator and risk of myocardial infarction. Lancet 1993, 341(8854):1165-1168.

55. Thogersen AM, Jansson JH, Boman K, Nilsson TK, Weinehall L, Huhtasaari F, Hallmans G: High plasminogen activator inhibitor and tissue plasminogen activator levels in plasma precede a first acute myocardial infarction in both men and women: evidence for the fibrinolytic system as an independent primary risk factor. Circulation 1998, 98(21):2241-2247.

56. Eliasson M, Evrin P, Lundblad D, Asplund K, Ranby M: Influence of gender, age and sampling time on plasma fibrinolytic variables and fibrinogen. Fibrinolysis 1993, 7:316-323.

57. Eliasson M, Jansson JH, Nilsson P, Asplund K: Increased levels of tissue plasminogen activator antigen in essential hypertension. A population-based study in Sweden. J Hypertens 1997, 15(4):349-356.

58. Jern S, Selin L, Bergbrant A, Jern C: Release of tissue-type plasminogen activator in response to muscarinic receptor stimulation in human forearm. Thromb Haemost 1994, 72(4):588-594.

59. Wall U: In vivo studies on local release of tissue-type plasminogen activator from vascular endothelium. Thesis Göteborg: Göteborg University 1997.

60. Chandler WL, Alessi MC, Aillaud MF, Henderson P, Vague P, Juhan-Vague I: Clearance of tissue plasminogen activator (TPA) and TPA/plasminogen activator inhibitor type 1 (PAI-1) complex: relationship to elevated TPA antigen in patients with high PAI-1 activity levels. Circulation 1997, 96(3):761-768.

61. Otter M, Kuiper J, van Berkel TJ, Rijken DC: Mechanisms of tissue-type plasminogen activator (tPA) clearance by the liver. Ann N Y Acad Sci 1992, 667:431-442.

62. Wall U, Jern C, Bergbrant A, Jern S: Enhanced levels of tissue-type plasminogen activator in borderline hypertension. Hypertension 1995, 26(5):796-800.

63. Benham FJ, Spurr N, Povey S, Brinton BT, Goodfellow PN, Solomon E, Harris TJ: Assignment of tissue-type plasminogen activator to chromosome 8 in man and identification of a common restriction length polymorphism within the gene. Mol Biol Med 1984, 2(4):251-259.

64. Rajput B, Degen SF, Reich E, Waller EK, Axelrod J, Eddy RL, Shows TB: Chromosomal locations of human tissue plasminogen activator and urokinase genes. Science 1985, 230(4726):672-674.

65. Degen SJ, Rajput B, Reich E: The human tissue plasminogen activator gene. J Biol Chem 1986, 261(15):6972-6985.

66. Fisher R, Waller EK, Grossi G, Thompson D, Tizard R, Schleuning WD: Isolation and characterization of the human tissue-type plasminogen activator structural gene including its 5' flanking region. J Biol Chem 1985, 260(20):11223-11230.

67. Levin EG: Gene expression and function of tissue type plasminogen activator. In: Glas-Greenwald P, editor Fibrinolysis in disease (Part I) Boca Raton: CRC Press 1995:p. 1-9.

68. Henderson BR, Sleigh MJ: TATA box-independent transcription of the human tissue plasminogen activator gene initiates within a sequence conserved in related genes. FEBS Lett 1992, 309(2):130-134.

69. Costa M, Shen Y, Maurer F, Medcalf RL: Transcriptional regulation of the tissue-type plasminogen-activator gene in human endothelial cells: identification of nuclear factors that recognise functional elements in the tissue-type plasminogen-activator gene promoter. Eur J Biochem 1998, 258(1):123-131.

70. Arts J, Herr I, Lansink M, Angel P, Kooistra T: Cell-type specific DNA-protein interactions at the tissue-type plasminogen activator promoter in human endothelial and HeLa cells in vivo and in vitro. Nucleic Acids Res 1997, 25(2):311-317.

71. Costa M, Medcalf RL: Differential binding of cAMP-responsive-element (CRE)-binding protein-1 and activating transcription factor-2 to a CRE-like element in the human tissue-type plasminogen activator (t-PA) gene promoter correlates with opposite regulation of t-PA by phorbol ester in HT-1080 and HeLa cells. Eur J Biochem 1996, 237(3):532-538.

72. Lux W, Klobeck HG, Daniel PB, Costa M, Medcalf RL, Schleuning WD: In vivo and in vitro analysis of the human tissue-type plasminogen activator gene promoter in neuroblastomal cell lines: evidence for a functional upstream kappaB element. J Thromb Haemost 2005, 3(5):1009-1017.

73. Resnick N, Collins T, Atkinson W, Bonthron DT, Dewey CF, Jr., Gimbrone MA, Jr.: Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear-stress-responsive element. Proc Natl Acad Sci U S A 1993, 90(10):4591-4595.

74. Bulens F, Merchiers P, Ibanez-Tallon I, De Vriese A, Nelles L, Claessens F, Belayew A, Collen D: Identification of a multihormone responsive enhancer far upstream from the human tissue-type plasminogen activator gene. J Biol Chem 1997, 272(1):663-671.

75. Booth NA, Simpson AJ, Croll A, Bennett B, MacGregor IR: Plasminogen activator inhibitor (PAI-1) in plasma and platelets. Br J Haematol 1988, 70(3):327-333.

76. Declerck PJ, Alessi MC, Verstreken M, Kruithof EK, Juhan-Vague I, Collen D: Measurement of plasminogen activator inhibitor 1 in biologic fluids with a murine monoclonal antibody-based enzyme-linked immunosorbent assay. Blood 1988, 71(1):220-225.

77. Juhan-Vague I, Pyke SD, Alessi MC, Jespersen J, Haverkate F, Thompson SG: Fibrinolytic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. ECAT Study Group. European Concerted Action on Thrombosis and Disabilities. Circulation 1996, 94(9):2057-2063.

78. Booth NA: Fibrinolysis and thrombosis. Baillieres Best Pract Res Clin Haematol 1999, 12(3):423-433.

79. Torr-Brown SR, Sobel BE: Attenuation of thrombolysis by release of plasminogen activator inhibitor type-1 from platelets. Thromb Res 1993, 72(5):413-421.

80. Huber K: Plasminogen activator inhibitor type-1 (part one): basic mechanisms, regulation, and role for thromboembolic disease. J Thromb Thrombolysis 2001, 11(3):183-193.

81. Alessi MC, Juhan-Vague I: PAI-1 and the metabolic syndrome: links, causes, and consequences. Arterioscler Thromb Vasc Biol 2006, 26(10):2200-2207.

82. Juhan-Vague I, Alessi MC, Mavri A, Morange PE: Plasminogen activator inhibitor-1, inflammation, obesity, insulin resistance and vascular risk. J Thromb Haemost 2003, 1(7):1575-1579.

83. Willerson JT, Ridker PM: Inflammation as a cardiovascular risk factor. Circulation 2004, 109(21 Suppl 1):II2-10.

84. Davies PF: Flow-mediated endothelial mechanotransduction. Physiol Rev 1995, 75(3):519-560.

85. Malek AM, Izumo S: Molecular aspects of signal transduction of shear stress in the endothelial cell. J Hypertens 1994, 12(9):989-999.

86. Asakura T, Karino T: Flow patterns and spatial distribution of atherosclerotic lesions in human coronary arteries. Circ Res 1990, 66(4):1045-1066.

87. Ku DN, Giddens DP, Zarins CK, Glagov S: Pulsatile flow and atherosclerosis in the human carotid bifurcation. Positive correlation between plaque location and low oscillating shear stress. Arteriosclerosis 1985, 5(3):293-302.

88. Glagov S: Intimal hyperplasia, vascular modeling, and the restenosis problem. Circulation 1994, 89(6):2888-2891.

89. Tronc F, Wassef M, Esposito B, Henrion D, Glagov S, Tedgui A: Role of NO in flow-induced remodeling of the rabbit common carotid artery. Arterioscler Thromb Vasc Biol 1996, 16(10):1256-1262.

90. Ali MH, Schumacker PT: Endothelial responses to mechanical stress: where is the mechanosensor? Crit Care Med 2002, 30(5 Suppl):S198-206.

91. Resnick N, Yahav H, Shay-Salit A, Shushy M, Schubert S, Zilberman LC, Wofovitz E: Fluid shear stress and the vascular endothelium: for better and for worse. Prog Biophys Mol Biol 2003, 81(3):177-199.

92. Lehoux S, Castier Y, Tedgui A: Molecular mechanisms of the vascular responses to haemodynamic forces. J Intern Med 2006, 259(4):381-392.

93. Dobrin PB: Mechanical properties of arteries. Physiol Rev 1978, 58(2):397-460.

94. Kakisis JD, Liapis CD, Sumpio BE: Effects of cyclic strain on vascular cells. Endothelium 2004, 11(1):17-28.

95. Lehoux S, Tedgui A: Cellular mechanics and gene expression in blood vessels. J Biomech 2003, 36(5):631-643.

96. Li YS, Haga JH, Chien S: Molecular basis of the effects of shear stress on vascular endothelial cells. J Biomech 2005, 38(10):1949-1971.

97. Lehoux S, Tedgui A: Signal transduction of mechanical stresses in the vascular wall. Hypertension 1998, 32(2):338-345.

98. Chang L, Karin M: Mammalian MAP kinase signalling cascades. Nature 2001, 410(6824):37-40.

99. Hoefen RJ, Berk BC: The role of MAP kinases in endothelial activation. Vascul Pharmacol 2002, 38(5):271-273.

100. Du W, Mills I, Sumpio BE: Cyclic strain causes heterogeneous induction of transcription factors, AP-1, CRE binding protein and NF-kB, in endothelial cells: species and vascular bed diversity. J Biomech 1995, 28(12):1485-1491.

101. Illi B, Nanni S, Scopece A, Farsetti A, Biglioli P, Capogrossi MC, Gaetano C: Shear stress-mediated chromatin remodeling provides molecular basis for flow-dependent regulation of gene expression. Circ Res 2003, 93(2):155-161.

102. Khachigian LM, Anderson KR, Halnon NJ, Gimbrone MA, Jr., Resnick N, Collins T: Egr-1 is activated in endothelial cells exposed to fluid shear stress and interacts with a novel shear-stress-response element in the PDGF A-chain promoter. Arterioscler Thromb Vasc Biol 1997, 17(10):2280-2286.

103. Lan Q, Mercurius KO, Davies PF: Stimulation of transcription factors NF kappa B and AP1 in endothelial cells subjected to shear stress. Biochem Biophys Res Commun 1994, 201(2):950-956.

104. Lin MC, Almus-Jacobs F, Chen HH, Parry GC, Mackman N, Shyy JY, Chien S: Shear stress induction of the tissue factor gene. J Clin Invest 1997, 99(4):737-744.

105. Wilson E, Vives F, Collins T, Ives HE: Strain-responsive regions in the platelet-derived growth factor-A gene promoter. Hypertension 1998, 31(1 Pt 2):170-175.

106. Iba T, Shin T, Sonoda T, Rosales O, Sumpio BE: Stimulation of endothelial secretion of tissue-type plasminogen activator by repetitive stretch. J Surg Res 1991, 50(5):457-460.

107. Carosi JA, McIntire LV: Effects of cyclical strain on the production of vasoactive materials by cultured human and bovine endothelial cells. European Respiratory Review 1993, 3(16):598-608.

108. Carosi JA, McIntire LV, Eskin SG: Modulation of secretion of vasoactive materials from human and bovine endothelial cells by cyclic strain. Biotechnology and bioengineering 1994, 43:615-621.

109. Iba T, Sumpio BE: Tissue plasminogen activator expression in endothelial cells exposed to cyclic strain in vitro. Cell Transplant 1992, 1(1):43-50.

110. Diamond SL, Eskin SG, McIntire LV: Fluid flow stimulates tissue plasminogen activator secretion by cultured human endothelial cells. Science 1989, 243(4897):1483-1485.

111. Diamond SL, Sharefkin JB, Dieffenbach C, Frasier-Scott K, McIntire LV, Eskin SG: Tissue plasminogen activator messenger RNA levels increase in cultured human endothelial cells exposed to laminar shear stress. J Cell Physiol 1990, 143(2):364-371.

112. Kawai Y, Matsumoto Y, Watanabe K, Yamamoto H, Satoh K, Murata M, Handa M, Ikeda Y: Hemodynamic forces modulate the effects of cytokines on fibrinolytic activity of endothelial cells. Blood 1996, 87(6):2314-2321.

113. Malek AM, Jackman R, Rosenberg RD, Izumo S: Endothelial expression of thrombomodulin is reversibly regulated by fluid shear stress. Circ Res 1994, 74(5):852-860.

114. Sjogren LS, Gan L, Doroudi R, Jern C, Jungersten L, Jern S: Fluid shear stress increases the intra-cellular storage pool of tissue-type plasminogen activator in intact human conduit vessels. Thromb Haemost 2000, 84(2):291-298.

115. Esmon CT: The interactions between inflammation and coagulation. Br J Haematol 2005, 131(4):417-430.

116. del Rincon ID, Williams K, Stern MP, Freeman GL, Escalante A: High incidence of cardiovascular events in a rheumatoid arthritis cohort not explained by traditional cardiac risk factors. Arthritis Rheum 2001, 44(12):2737-2745.

117. Goodson NJ, Wiles NJ, Lunt M, Barrett EM, Silman AJ, Symmons DP: Mortality in early inflammatory polyarthritis: cardiovascular mortality is increased in seropositive patients. Arthritis Rheum 2002, 46(8):2010-2019.

118. Jacobsson LT, Turesson C, Gulfe A, Kapetanovic MC, Petersson IF, Saxne T, Geborek P: Treatment with tumor necrosis factor blockers is associated with a lower incidence of first cardiovascular events in patients with rheumatoid arthritis. J Rheumatol 2005, 32(7):1213-1218.

119. Pober JS, Cotran RS: Cytokines and endothelial cell biology. Physiol Rev 1990, 70(2):427-451.

120. Madge LA, Pober JS: TNF signaling in vascular endothelial cells. Exp Mol Pathol 2001, 70(3):317-325.

121. Pober JS: Endothelial activation: intracellular signaling pathways. Arthritis Res 2002, 4 Suppl 3:S109-116.

122. Baud V, Karin M: Signal transduction by tumor necrosis factor and its relatives. Trends Cell Biol 2001, 11(9):372-377.

123. Karin M, Liu Z, Zandi E: AP-1 function and regulation. Curr Opin Cell Biol 1997, 9(2):240-246.

124. Kyriakis JM: Activation of the AP-1 transcription factor by inflammatory cytokines of the TNF family. Gene Expr 1999, 7(4-6):217-231.

125. Muth H, Maus U, Wygrecka M, Lohmeyer J, Grimminger F, Seeger W, Gunther A: Pro- and antifibrinolytic properties of human pulmonary microvascular versus artery endothelial cells: impact of endotoxin and tumor necrosis factor-alpha. Crit Care Med 2004, 32(1):217-226.

126. Nachman RL, Hajjar KA, Silverstein RL, Dinarello CA: Interleukin 1 induces endothelial cell synthesis of plasminogen activator inhibitor. J Exp Med 1986, 163(6):1595-1600.

127. Schleef RR, Bevilacqua MP, Sawdey M, Gimbrone MA, Jr., Loskutoff DJ: Cytokine activation of vascular endothelium. Effects on tissue-type plasminogen activator and type 1 plasminogen activator inhibitor. J Biol Chem 1988, 263(12):5797-5803.

128. van Hinsbergh VW, Kooistra T, van den Berg EA, Princen HM, Fiers W, Emeis JJ: Tumor necrosis factor increases the production of plasminogen activator inhibitor in human endothelial cells in vitro and in rats in vivo. Blood 1988, 72(5):1467-1473.

129. Bevilacqua MP, Schleef RR, Gimbrone MA, Jr., Loskutoff DJ: Regulation of the fibrinolytic system of cultured human vascular endothelium by interleukin 1. J Clin Invest 1986, 78(2):587-591.

130. Chia S, Qadan M, Newton R, Ludlam CA, Fox KA, Newby DE: 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(4):695-701.

131. van Hinsbergh VW, Bauer KA, Kooistra T, Kluft C, Dooijewaard G, Sherman ML, Nieuwenhuizen W: Progress of fibrinolysis during tumor necrosis factor infusions in humans. Concomitant increase in tissue-type plasminogen activator, plasminogen activator inhibitor type-1, and fibrin(ogen) degradation products. Blood 1990, 76(11):2284-2289.

132. Jaffe EN, RL. Becker, CG. Minutesick, CR.: Culture of human endothelial cells derived from umbilical cord veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973, 52:2745-2756.

133. Higuchi R, Fockler C, Dollinger G, Watson R: Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology (N Y) 1993, 11(9):1026-1030.

134. Osborn L, Kunkel S, Nabel GJ: Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. Proc Natl Acad Sci U S A 1989, 86(7):2336-2340.

135. Sambrock J, Fritsch E, Maniatis T: Molecular cloning: a laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory. 1989, 2nd edition.

136. Bergh N, Ekman M, Ulfhammer E, Andersson M, Karlsson L, Jern S: A new biomechanical perfusion system for ex vivo study of small biological intact vessels. Ann Biomed Eng 2005, 33(12):1808-1818.

137. Redmond EM, Cullen JP, Cahill PA, Sitzmann JV, Stefansson S, Lawrence DA, Okada SS: Endothelial cells inhibit flow-induced smooth muscle cell migration: role of plasminogen activator inhibitor-1. Circulation 2001, 103(4):597-603.

138. Jern C, Seeman-Lodding H, Biber B, Winso O, Jern S: An experimental multiple-organ model for the study of regional net release/uptake rates of tissue-type plasminogen activator in the intact pig. Thromb Haemost 1997, 78(3):1150-1156.

139. Wall U, Jern S, Tengborn L, Jern C: Evidence of a local mechanism for desmopressin-induced tissue-type plasminogen activator release in human forearm. Blood 1998, 91(2):529-537.

140. Minai K, Matsumoto T, Horie H, Ohira N, Takashima H, Yokohama H, Kinoshita M: Bradykinin stimulates the release of tissue plasminogen activator in human coronary circulation: effects of angiotensin-converting enzyme inhibitors. J Am Coll Cardiol 2001, 37(6):1565-1570.

141. Lip GY, Blann AD: Does hypertension confer a prothrombotic state? Virchow's triad revisited. Circulation 2000, 101(3):218-220.

142. Brown NJ, Agirbasli M, Vaughan DE: Comparative effect of angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor antagonism on plasma fibrinolytic balance in humans. Hypertension 1999, 34(2):285-290.

143. Sakata K, Shirotani M, Yoshida H, Urano T, Takada Y, Takada A: Differential effects of enalapril and nitrendipine on the fibrinolytic system in essential hypertension. Am Heart J 1999, 137(6):1094-1099.

144. de Boer A, Kluft C, Kroon JM, Kasper FJ, Schoemaker HC, Pruis J, Breimer DD, Soons PA, Emeis JJ, Cohen AF: Liver blood flow as a major determinant of the clearance of recombinant human tissue-type plasminogen activator. Thromb Haemost 1992, 67(1):83-87.

145. Matsumoto T, Minai K, Horie H, Ohira N, Takashima H, Tarutani Y, Yasuda Y, Ozawa T, Matsuo S, Kinoshita M et al: Angiotensin-converting enzyme inhibition but not angiotensin II type 1 receptor antagonism augments coronary release of tissue plasminogen activator in hypertensive patients. J Am Coll Cardiol 2003, 41(8):1373-1379.

146. Labinjoh C, Newby DE, Pellegrini MP, Johnston NR, Boon NA, Webb DJ: Potentiation of bradykinin-induced tissue plasminogen activator release by angiotensin-converting enzyme inhibition. J Am Coll Cardiol 2001, 38(5):1402-1408.

147. Pretorius M, Luther JM, Murphey LJ, Vaughan DE, Brown NJ: Angiotensin-converting enzyme inhibition increases basal vascular tissue plasminogen activator release in women but not in men. Arterioscler Thromb Vasc Biol 2005, 25(11):2435-2440.

148. Pretorius M, Rosenbaum D, Vaughan DE, Brown NJ: Angiotensin-converting enzyme inhibition increases human vascular tissue-type plasminogen activator release through endogenous bradykinin. Circulation 2003, 107(4):579-585.

149. Ghiadoni L, Magagna A, Versari D, Kardasz I, Huang Y, Taddei S, Salvetti A: Different effect of antihypertensive drugs on conduit artery endothelial function. Hypertension 2003, 41(6):1281-1286.

150. Taddei S, Virdis A, Ghiadoni L, Mattei P, Salvetti A: Effects of angiotensin converting enzyme inhibition on endothelium-dependent vasodilatation in essential hypertensive patients. J Hypertens 1998, 16(4):447-456.

151. On YK, Kim CH, Oh BH, Lee MM, Park YB: Effects of angiotensin converting enzyme inhibitor and calcium antagonist on endothelial function in patients with essential hypertension. Hypertens Res 2002, 25(3):365-371.

152. Perticone F, Ceravolo R, Maio R, Ventura G, Iacopino S, Cuda G, Mastroroberto P, Chello M, Mattioli PL: Calcium antagonist isradipine improves abnormal endothelium-dependent vasodilation in never treated hypertensive patients. Cardiovasc Res 1999, 41(1):299-306.

153. Taddei S, Virdis A, Ghiadoni L, Uleri S, Magagna A, Salvetti A: Lacidipine restores endothelium-dependent vasodilation in essential hypertensive patients. Hypertension 1997, 30(6):1606-1612.

154. von zur Muhlen B, Kahan T, Hagg A, Millgard J, Lind L: Treatment with irbesartan or atenolol improves endothelial function in essential hypertension. J Hypertens 2001, 19(10):1813-1818.

155. Cheng JJ, Chao YJ, Wung BS, Wang DL: Cyclic strain-induced plasminogen activator inhibitor-1 (PAI-1) release from endothelial cells involves reactive oxygen species. Biochem Biophys Res Commun 1996, 225(1):100-105.

156. Poli KA, Tofler GH, Larson MG, Evans JC, Sutherland PA, Lipinska I, Mittleman MA, Muller JE, D'Agostino RB, Wilson PW et al: Association of blood pressure with fibrinolytic potential in the Framingham offspring population. Circulation 2000, 101(3):264-269.

157. Sumpio BE, Chang R, Xu WJ, Wang XJ, Du W: Regulation of tPA in endothelial cells exposed to cyclic strain: role of CRE, AP-2, and SSRE binding sites. Am J Physiol 1997, 273(5 Pt 1):C1441-1448.

158. McCormick SM, Eskin SG, McIntire LV, Teng CL, Lu CM, Russell CG, Chittur KK: DNA microarray reveals changes in gene expression of shear stressed human umbilical vein endothelial cells. Proc Natl Acad Sci U S A 2001, 98(16):8955-8960.

159. Mohan S, Mohan N, Valente AJ, Sprague EA: Regulation of low shear flow-induced HAEC VCAM-1 expression and monocyte adhesion. Am J Physiol 1999, 276(5 Pt 1): C1100-1107.

160. Sampath R, Kukielka GL, Smith CW, Eskin SG, McIntire LV: Shear stress-mediated changes in the expression of leukocyte adhesion receptors on human umbilical vein endothelial cells in vitro. Ann Biomed Eng 1995, 23(3):247-256.

161. Uematsu M, Ohara Y, Navas JP, Nishida K, Murphy TJ, Alexander RW, Nerem RM, Harrison DG: Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress. Am J Physiol 1995, 269(6 Pt 1):C1371-1378.

162. Kadohama T, Akasaka N, Nishimura K, Hoshino Y, Sasajima T, Sumpio BE: p38 Mitogen-activated protein kinase activation in endothelial cell is implicated in cell alignment and elongation induced by fluid shear stress. Endothelium 2006, 13(1):43-50.

163. Traub O, Berk BC: Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. Arterioscler Thromb Vasc Biol 1998, 18(5):677-685.

164. Bautista LE, Lopez-Jaramillo P, Vera LM, Casas JP, Otero AP, Guaracao AI: Is C-reactive protein an independent risk factor for essential hypertension? J Hypertens 2001, 19(5):857-861.

165. Bautista LE, Vera LM, Arenas IA, Gamarra G: Independent association between inflammatory markers (C-reactive protein, interleukin-6, and TNF-alpha) and essential hypertension. J Hum Hypertens 2005, 19(2):149-154.

166. Blake GJ, Rifai N, Buring JE, Ridker PM: Blood pressure, C-reactive protein, and risk of future cardiovascular events. Circulation 2003, 108(24):2993-2999.

167. Chae CU, Lee RT, Rifai N, Ridker PM: Blood pressure and inflammation in apparently healthy men. Hypertension 2001, 38(3):399-403.

168. Sesso HD, Buring JE, Rifai N, Blake GJ, Gaziano JM, Ridker PM: C-reactive protein and the risk of developing hypertension. Jama 2003, 290(22):2945-2951.

169. Mawatari M, Okamura K, Matsuda T, Hamanaka R, Mizoguchi H, Higashio K, Kohno K, Kuwano M: Tumor necrosis factor and epidermal growth factor modulate migration of human microvascular endothelial cells and production of tissue-type plasminogen activator and its inhibitor. Exp Cell Res 1991, 192(2):574-580.

170. Gingras D, Nyalendo C, Di Tomasso G, Annabi B, Beliveau R: Activation of tissue plasminogen activator gene transcription by Neovastat, a multifunctional antiangiogenic agent. Biochem Biophys Res Commun 2004, 320(1):205-212.

171. Anderson HD, Rahmutula D, Gardner DG: Tumor necrosis factor-alpha inhibits endothelial nitric-oxide synthase gene promoter activity in bovine aortic endothelial cells. J Biol Chem 2004, 279(2):963-969.

172. Sohn RH, Deming CB, Johns DC, Champion HC, Bian C, Gardner K, Rade JJ: Regulation of endothelial thrombomodulin expression by inflammatory cytokines is mediated by activation of nuclear factor-kappa B. Blood 2005, 105(10):3910-3917.

173. May MJ, Ghosh S: Rel/NF-kappa B and I kappa B proteins: an overview. Semin Cancer Biol 1997, 8(2):63-73.

174. Kumar A, Lin Z, SenBanerjee S, Jain MK: Tumor necrosis factor alpha-mediated reduction of KLF2 is due to inhibition of MEF2 by NF-kappaB and histone deacetylases. Mol Cell Biol 2005, 25(14):5893-5903.

175. Gustin JA, Pincheira R, Mayo LD, Ozes ON, Kessler KM, Baerwald MR, Korgaonkar CK, Donner DB: Tumor necrosis factor activates CRE-binding protein through a p38 MAPK/MSK1 signaling pathway in endothelial cells. Am J Physiol Cell Physiol 2004, 286(3):C547-555.

176. Saccani S, Pantano S, Natoli G: p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment. Nat Immunol 2002, 3(1):69-75.

177. Robinson SD, Dawson P, Ludlam CA, Boon NA, Newby DE: Vascular and fibrinolytic effects of intra-arterial tumour necrosis factor-alpha in patients with coronary heart disease. Clin Sci (Lond) 2006, 110(3):353-360.

178. van der Poll T, Levi M, Buller HR, van Deventer SJ, de Boer JP, Hack CE, ten Cate JW: Fibrinolytic response to tumor necrosis factor in healthy subjects. J Exp Med 1991, 174(3):729-732.

179. Speidl WS, Zeiner A, Nikfardjam M, Geppert A, Jordanova N, Niessner A, Zorn G, Maurer G, Schreiber W, Wojta J et al: An increase of C-reactive protein is associated with enhanced activation of endogenous fibrinolysis at baseline but an impaired endothelial fibrinolytic response after venous occlusion. J Am Coll Cardiol 2005, 45(1):30-34.

180. Dandona P, Aljada A, Chaudhuri A, Mohanty P, Garg R: Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation. Circulation 2005, 111(11):1448-1454.

181. Boos CJ, Lip GY: Is hypertension an inflammatory process? Curr Pharm Des 2006, 12(13):1623-1635.

182. Verma S, Wang CH, Li SH, Dumont AS, Fedak PW, Badiwala MV, Dhillon B, Weisel RD, Li RK, Mickle DA et al: A self-fulfilling prophecy: C-reactive protein attenuates nitric oxide production and inhibits angiogenesis. Circulation 2002, 106(8):913-919.

183. Intengan HD, Schiffrin EL: Vascular remodeling in hypertension: roles of apoptosis, inflammation, and fibrosis. Hypertension 2001, 38(3 Pt 2):581-587.

184. Duprez DA, Somasundaram PE, Sigurdsson G, Hoke L, Florea N, Cohn JN: Relationship between C-reactive protein and arterial stiffness in an asymptomatic population. J Hum Hypertens 2005, 19(7):515-519.

185. Giannarelli C, De Negri F, Virdis A, Ghiadoni L, Cipriano A, Magagna A, Taddei S, Salvetti A: Nitric oxide modulates tissue plasminogen activator release in normotensive subjects and hypertensive patients. Hypertension 2007, 49(4):878-884.

186. Singh U, Devaraj S, Jialal I: C-reactive protein decreases tissue plasminogen activator activity in human aortic endothelial cells: evidence that C-reactive protein is a procoagulant. Arterioscler Thromb Vasc Biol 2005, 25(10):2216-2221.