Pharmacological stimulation of endothelial function and long-term impact of hypertension in man

Ott Saluveer
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To My Family
ABSTRACT

Background: Ischemic heart disease is a major cause of death globally. Rupture of a coronary atherosclerotic plaque with occluding thrombus formation is the main cause of myocardial ischemia and infarction. A healthy vascular endothelium is pivotal for maintenance of vessel patency and normal blood flow, which is important for prevention of thrombotic events. In the event of an intra-arterial thrombosis formation the endothelium reacts with vasodilatation and activation of the endogenous fibrinolytic system. Endothelial dysfunction (ED) is a common denominator in patients with different cardiovascular risk factors including hypertension. ED promotes a vasoconstrictive, prothrombotic, and proinflammatory state. ED in hypertension is associated with impaired endothelium-dependent vasodilation (EDV) and impaired endogenous fibrinolysis measured as acute stimulated tissue plasminogen activator (t-PA) release. Hypertension confers a prothrombotic state and ED could be an important contributor to the increased risk for atherothrombotic events.

Aims: The overall aim of this thesis was to pharmacologically improve endothelial function in hypertension and normotension, and to investigate the long-term prognostic impact of hypertension. The aim of Study I-II was to investigate if pharmacological intervention by atorvastatin (ATV) or sodium nitroprusside (SNP) may improve vascular function in terms of EDV or fibrinolytic capacity, respectively, in hypertensive men. The aim of Study III was to evaluate if histone deacetylase inhibition by valproic acid (VPA) affects the endogenous fibrinolytic system, measured as t-PA release capacity or plasminogen activator inhibitor-1 (PAI-I) levels in a cohort of healthy men. The aim of Study IV was to investigate the long-term prognostic impact of hypertension on the mortality after percutaneous coronary intervention (PCI).

Methods: In the clinical experimental studies, venous occlusion plethysmography and intra-brachial infusion of vasoactive substances were used to assess endothelium-dependent vasodilation (EDV), and endothelium-independent vasodilation (EIDV) or vasoconstriction responses in the forearm (Studies I-III). The perfused forearm model was used to measure stimulated t-PA release capacity (Studies II-III) in the forearm. t-PA Release was stimulated by intra-brachial infusion of Substance P. Long-term prognostic impact of hypertension on total mortality after PCI was investigated in a large register study using the Swedish Coronary Angiography and Angioplasty Register (SCAAR), in which data were analyzed for 175,892 patients.

Results: ATV treatment did not improve EDV acutely in hypertensive men. Forearm vascular resistance in response to SNP was lowered by ATV, and vasoconstriction in response to Angiotensin II (Ang II) was diminished by ATV treatment. Acute blood pressure lowering by SNP did not affect Substance P induced t-PA release capacity in patients with hypertension. VPA treatment resulted in considerably decreased levels of circulating PAI-1 antigen, and the t-PA:PAI-1 antigen ratio increased. Acute t-PA release in response to Substance P was not affected by VPA. The SCAAR-study showed that hypertension is associated with higher mortality risk in patients undergoing PCI in Sweden, and the risk was highest in patients less than 65 years, in smokers and in patients with ST-elevation myocardial infarction (STEMI).

Conclusions: The observed acute statin effects in hypertension seem to be endothelium-independent and related to vascular smooth muscle cell function. Acute blood pressure lowering does not restore the impaired fibrinolytic capacity in hypertension, suggesting a diminished releasable t-PA pool in the endothelium. Intervention by VPA treatment did not affect the acute stimulated t-PA release capacity in healthy man. In contrary, VPA diminished plasma PAI-1 antigen levels and altered the fibrinolytic balance, measured as t-PA:PAI-1 ratio in a profibrinolytic direction. Further studies are needed to confirm fibrinolytic effects of histone deacetylase inhibitors in patients with ED, e.g. established atherosclerosis. A long-term adverse impact of hypertension diagnosis on survival after PCI was demonstrated in a large-scale register study, and the highest risk was found in patients with STEMI. These findings underscore the importance of optimal secondary prevention including blood pressure control in patients with coronary artery disease.

Keywords: t-PA, hypertension, fibrinolysis, endothelial function, valproic acid, histone deacetylase inhibitor, atorvastatin, percutaneous coronary intervention, acute coronary syndromes

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LIST OF PAPERS

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals.


Study IV: Hypertension is associated with increased mortality in patients with acute coronary syndromes after revascularization with percutaneous coronary intervention – a report from SCAAR. Ott Saluveer, Björn Redfors, Oskar Angerås, Christian Dworeck, Inger Haraldsson, Petur Petursson, Jacob Odenstedt, Dan Ioanes, Peter Lundgren, Sebastian Völz, Truls Råmunddal, Bert Andersson, Elmir Omerovic, Niklas Bergh. *Submitted*
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<td>ACS</td>
<td>Acute coronary syndromes</td>
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<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
<td>CABG</td>
<td>Coronary artery bypass grafting</td>
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<td>CAD</td>
<td>Coronary artery disease</td>
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<td>CONSORT</td>
<td>Consolidated standards of reporting trials</td>
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<td>CVD</td>
<td>Cardiovascular disease</td>
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<td>CV</td>
<td>Cardiovascular</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>ECG</td>
<td>Electrocardiography</td>
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<td>ED</td>
<td>Endothelial dysfunction</td>
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<td>EDV</td>
<td>Endothelium-dependent vasodilation</td>
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<td>EIDV</td>
<td>Endothelium-independent vasodilation</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>EudraCT</td>
<td>European union drug regulating authorities clinical trials</td>
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<td>FBF</td>
<td>Forearm blood flow</td>
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<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
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<tr>
<td>Hs-CRP</td>
<td>High sensitive C-reactive protein</td>
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<td>IHD</td>
<td>Ischemic heart disease</td>
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<tr>
<td>LAD</td>
<td>Left anterior descending artery</td>
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<tr>
<td>min</td>
<td>Minutes</td>
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<tr>
<td>mL</td>
<td>Milliliter</td>
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<tr>
<td>ng</td>
<td>Nanograms</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<td>NSTEMI</td>
<td>Non-ST-elevation myocardial infarction</td>
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<td>PAI-1</td>
<td>Plasminogen activator inhibitor -1</td>
</tr>
<tr>
<td>PCI</td>
<td>Percutaneous coronary intervention</td>
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<tr>
<td>SCAAR</td>
<td>Swedish Coronary Angiography and Angioplasty Register</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>STEMI</td>
<td>ST-elevation myocardial infarction</td>
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<tr>
<td>t-PA</td>
<td>Tissue plasminogen activator</td>
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<tr>
<td>UA</td>
<td>Unstable angina</td>
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<td>VPA</td>
<td>Valproic acid</td>
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INTRODUCTION

Cardiovascular disease (CVD), in particular coronary artery disease (CAD) due to atherosclerosis, is a major cause of death and disability globally [1]. Rupture of a coronary atherosclerotic plaque with occluding thrombus formation is the main cause of myocardial ischemia and infarction. Without reduction of CVD risk factors, many countries will see an increase in CVD mortality in the future. In 2025, 7.8 million premature CVD deaths are estimated, if current risk factor trends continue [2].

A healthy vascular endothelium is pivotal for maintenance of vessel patency and normal blood flow, protecting humans from thrombotic events [3]. In the event of an intra-arterial thrombosis formation the endothelium reacts with vasodilation and activation of the endogenous fibrinolytic system in order to restore blood flow and to dissolve the clot. These acute endothelial responses are impaired and insufficient for thromboprotection in humans when endothelial dysfunction (ED) is present. ED is characterized by impaired vascular homeostasis of physiological vasoprotective mechanisms, resulting in a vasoconstrictive, proinflammatory and prothrombotic state [4]. The mostly frequently studied feature of ED in man is the impaired endothelium-dependent vasodilation (EDV) depending on reduced nitric oxide (NO) bioavailability. Another clinically relevant aspect of ED is the capacity of endogenous fibrinolysis. Impaired EDV and endogenous fibrinolysis are common denominators in patients with different cardiovascular risk factors including hypertension [5,6], and coronary atherosclerosis [7]. ED is an independent predictor of cardiovascular events in addition to traditional risk factors [8,9]. Acting as an initiator of the atherosclerotic process, ED plays a role in the development of CVD, and it could also be an important factor in the progression of atherosclerosis [4,10], eventually leading to thrombotic events.

Pharmacologically targeting the endothelium to restore endothelial function could be a future interesting target in prevention and treatment of CAD.

The overall hypothesis underlying this thesis is that the increased risk for thrombosis in hypertensive patients partly depends on impaired endothelium-dependent vasodilation and impaired capacity for endogenous fibrinolysis.

ENDothelium

The vascular endothelium is an active monolayer of cells covering the lumen of all blood vessels. The endothelium is a metabolically active organ, and it is sensitive to mechanical, chemical and humoral stimuli. Normal endothelial function is characterized by homeostasis of various physiological functions [10]. The endothelium maintains a functional balance between release of vasodilatory, thrombolytic, anti-inflammatory, and anti-coagulant agents, and their pro-coagulant, vasoconstrictive, and thrombotic counterparts [3]. Vasodilation is mainly mediated by release of nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF), and prostacyclin, while vasoconstriction is mediated by factors such as endothelin-1 (ET-1), Angiotensin II (Ang II), thromboxane A2, and prostaglandin H2 [4]. NO is considered to be the most potent endogenous vasodilator in man, and NO also contributes to maintain vascular
homeostasis by inhibiting platelet aggregation, inflammation, oxidative stress, and vascular smooth muscle cell proliferation [4]. The endogenous fibrinolysis is mainly activated by regulated release of tissue plasminogen activator (t-PA) [11,12,13] from the endothelium. The main inhibitor of the endogenous fibrinolytic system is plasminogen activator inhibitor-1 (PAI-1) [14,15,16], which can complex-bind and thereby inactivate t-PA.

**Endothelial dysfunction**

Endothelial dysfunction (ED) is a major link between exposure to cardiovascular risk factors and the development of atherosclerotic disease. ED is characterized by decreased endothelial release of vasodilatory, thrombolytic, anti-inflammatory, and anti-coagulant molecules, relative to their vasoconstrictive, thrombotic, and inflammatory counterparts [17]. This dysfunctional balance promotes vasoconstriction, thrombosis, oxidation and inflammation. All the major risk factors for cardiovascular (CV) events have been associated with impaired endothelial nitric oxide (NO) activity [3], which is a primary marker for endothelial dysfunction [17,18]. Oxidative stress appears to be the most common underlying mechanism for the development of ED, and most CV risk factors are associated with up-regulation of oxidative stress and reactive oxygen species, which lead to reduced NO availability [19]. Reduced NO bioavailability promotes vasoconstriction, thrombosis, inflammation, platelet aggregation, lipid deposition, vascular smooth cell proliferation, and leukocyte adhesion [18].

A reduced EDV in the coronary circulation is an independent risk factor for CV events irrespective of presence or absence of angiographic coronary lesions [20,21]. ED measured as decreased EDV in forearm circulation is an independent predictor of CV mortality in CAD, including patients with acute coronary syndromes (ACS) [22,23]. In unstable angina abolition of postischemic vasodilation of the brachial artery has been demonstrated [17]. Further underscoring the importance of reversing ED in CAD is the finding that the recovery of EDV after ACS is associated with event-free survival [23].

**Hypertension and endothelial dysfunction**

Hypertension is a major risk factor for cardiovascular events [24,25], and blood pressure levels show an independent continuous relationship with the incidence of atherothrombotic events [24]. The major complications of hypertension are ischemic stroke, myocardial infarction, heart failure, peripheral artery disease, renal failure, and cardiovascular death [26]. The increased risk of ischemic events in hypertension has often been taken as an effect of accelerated atherosclerosis and emerging plaque rupture. Thus, hypertension confers a pro-thrombotic state [27].

Hypertension is characterized by ED, which is also a marker of future cardiovascular events in hypertension [28]. The main factor attributable to ED in hypertension is reduced or absent availability of NO [5], which is mainly considered to be a consequence of increased oxidative stress. In hypertension, endothelium-dependent contractions are pronounced [29], and the endothelium-dependent vasodilation is impaired [5].
ED in hypertension is also characterized by impaired capacity for endogenous fibrinolysis [6,30] in man, but the mechanisms behind this impairment are far from completely understood. Mechanical stress exerted by different mechanical forces on the endothelium by high blood pressure may contribute to induce ED. Previous studies have shown that increased intraluminal pressure down-regulates the expression of t-PA and decreases t-PA secretion from the endothelium in an ex vivo model [31]. Furthermore, prolonged high laminar shear stress has shown to be a major mechanical suppressor of t-PA in vitro [32], and shear stress has a more powerful down-regulatory effect on t-PA gene expression than tensile stress [33].

Pharmacological improvement of the impaired EDV in hypertensive patients can be achieved by different antihypertensive long-term treatment regimens. Angiotensin-converting enzyme inhibitors (ACEIs) improve endothelial function by inhibiting angiotensin-converting enzyme and reducing the production of angiotensin II (Ang II) [34]. ACEIs stabilize bradykinin, which induces the release of NO and prostacyclin, and ACEIs also reduce production of free radicals which is stimulated by Ang II [35]. Angiotensin receptor blockers (ARB) have also showed to improve endothelial function, supporting an important role of Ang II in the development of atherosclerosis [36].

The impaired fibrinolytic capacity in hypertension can be restored by long-term treatment with antihypertensive drugs [37,38]. This observation suggests that the improvement of fibrinolytic capacity may be related to a blood pressure reduction as such rather than a specific pharmacological effect [37]. ACEIs have also been shown to have a suppressive effect on PAI-1 levels, altering the fibrinolytic balance in favor of fibrinolysis [30], and they have also shown to reduce thrombin generation in hypertensive subjects [39]. Results from large placebo controlled trials of patients with ventricular dysfunction after myocardial infarction have shown a significant decrease in the incidence of coronary events in patients treated with ACEIs, irrespectively of its effect on blood pressure [30]. Thus, it is believed that the mechanisms behind reduction of the cardiovascular risk by antihypertensive drugs include improving endothelial fibrinolytic function [30] in both primary and secondary prevention.

**Long-term impact of hypertension**

Hypertension is highly prevalent in patients with established coronary artery disease [1]. Data about the prognostic role of hypertension on long-term survival in patients who are revascularized with percutaneous coronary intervention (PCI) are still limited and inconsistent. Studies with both neutral [40,41,42] and adverse outcome post PCI [43,44,45] have been reported. The influence of hypertension on prognosis after PCI has not been studied in larger patient cohorts.

**Statins and endothelial function**

Treatment with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) is widely used in primary and secondary prevention of cardiovascular disease [46,47,48]. Statins also have multiple effects independent of their cholesterol-lowering actions, generally named pleiotropic effects. In endothelial cells statins
are able to increase expression of endothelial nitric oxide synthases (eNOs), reduce oxidative stress, and they have anti-inflammatory and anti-thrombotic properties [49]. Effects of statins on reversing ED occur rapidly in vitro [50].

Studies have shown that EDV is impaired in patients with hypercholesterolemia [51,52,53], and a normalization of EDV has been demonstrated by long term use of different statins [54,55,56,57]. A meta-analysis has showed that statin therapy is associated with significant improvement of EDV in both peripheral and coronary endothelium [58]. A rapid initiation of statin therapy, regardless of the actual cholesterol level, could be of importance for the outcome of patients with ACS [59,60]. A meta-analysis has confirmed time-related benefits of statins in patients with ACS undergoing PCI [61]. This analysis showed that early statin administration before PCI correlated significantly with lower risk of major adverse CV events at 30 days. High-dose statins administered prior to PCI for ACS or stable CAD have shown to significantly decrease both short-, and long-term CV mortality compared with low-dose statins or no statins [62,63]. The mechanisms are considered to involve the pleiotropic effects of statins, including improved endothelial function, reduced inflammation and decreased thrombotic tendency. Regarding fibrinolysis, hypercholesterolemia or statin therapy have not been shown to affect the stimulated endogenous fibrinolytic capacity in vivo [64].

**The endogenous fibrinolytic system**

The endogenous fibrinolytic system is regulated by circulating factors and factors released from the endothelium. In case of an atherosclerotic plaque rupture (Figure 1), the endothelium reacts with a local fibrinolytic response initiated by a massive local release of tissue plasminogen activator (t-PA), in order to restore blood flow and to dissolve the clot. Agonists from the coagulation cascade stimulate the endothelial cells to release large amounts of free t-PA. Free t-PA converts the thrombus-bound proenzyme plasminogen to plasmin. Plasmin then degrades fibrin into fibrin degradation products, thus dissolving the thrombus. t-PA Induced activation of plasminogen is the physiologically most important trigger of fibrinolysis [65,66]. The main inhibitor of active t-PA is circulating PAI-1 [67,68]. Although PAI-1 is synthesized in endothelial cells in vitro [15], there is no releasable pool in the endothelium in vivo [14]. The main source of plasma PAI-1 is the platelets, that retain high levels of active PAI-1 [15,69,70].

**Tissue plasminogen activator**

t-PA Is a 527-amino glycoprotein with a molecular weight of 65-75 kD depending on its degree of glycosylation [71,72]. The human gene coding for t-PA is localized on chromosome 8 [71]. Recent research by our group and others has shown that the t-PA-gene expression is under epigenetic control, and several histone deacetylase (HDAC) inhibitors markedly upregulate t-PA-gene expression in vitro [73,74,75].

t-PA Is released into the circulation both through a constitutive and a regulated pathway [76]. Normally, t-PA is constitutively secreted at a low rate from the endothelium. A regulated release of t-PA occurs upon agonist stimulation of the endothelium, e.g. in
Figure 1. The endothelial fibrinolytic response to an evolving trombus. Agonists from the coagulation cascade (eg. thrombin, factor Xa) act on endothelial cell surface G-protein coupled receptors (GPCR) (1), to stimulate release of t-PA from storage granules via increase in intracellular calcium concentration (2). Free t-PA acts on thrombus-bound plasminogen (3), convert plasminogen to plasmin (4), that in turn degrades cross-linked fibrin into fibrin degradation products (FDPs)(5), thus dissolving the thrombus. The fibrinolytic process is inhibited by inactivation of t-PA by circulating PAI-1, and plasmin by α2-antiplasmin. Figure adapted from Oliver et al (Oliver JJ et al. ATVB 2005;25:2470-79).

case of an atherosclerotic plaque rupture (Figure 1) [13,76]. The most potent trigger of regulated t-PA release is a thrombotic event, when stimulation of the endothelial surface by products of the coagulation cascade (i.e. thrombin and Factor Xa) results in a release of t-PA from intracellular storage granules [13,77,78,79]. This leads to very high local concentration of t-PA in the vessel lumen, to protect the vessel from thrombotic occlusion. t-PA Present during thrombus generation, before the fibrin network is stabilized, results in a more effective fibrinolysis compared to when t-PA is added afterwards [80,81]. Besides a thrombotic event, also adrenergic agonists and local ischemia can stimulate t-PA release [82,83,84]. In addition, a number of endogenous and exogenous receptor agonists can stimulate t-PA release in vivo, including nor-ephinephrine, Substance P, bradykinin, desmopressin, metacholine, and acetylcholine [11,12,85,86].

t-PA and PAI-I as cardiovascular risk factors

There is a considerable body of evidence for impaired endogenous fibrinolytic system as an independent cardiovascular risk factor [87,88]. Genetic variations of the t-PA gene have been associated with forearm vascular release rates of t-PA [89,90]. A t-PA gene polymorphism associated with low basal secretion rate of t-PA is associated with increased risk of future adverse cardiovascular events [87,91]. Impaired t-PA release has been associated with several well-established risk factors for cardiovascular events, including hypertension [6,92], obesity [93], smoking and CAD [7,94]. Furthermore, the impaired t-PA release predicts adverse cardiovascular events in patients with CAD [95].
Both t-PA and PAI-1 levels are risk factors for a first myocardial infarction [88,96,97,98]. They are also significant risk markers for recurrent myocardial infarction [99,100]. However, it is not obvious why elevated t-PA antigen levels are associated with coronary events, since t-PA is a profibrinolytic enzyme. There are alternative explanations to explain this enigma. Most of the t-PA in plasma is complex bound to PAI-1 during baseline conditions [101], and there is a strong association between PAI-1 and t-PA antigen levels [102]. Baseline t-PA antigen is in part a surrogate measure of PAI-I level [102]. t-PA Levels may also reflect the acute phase response in CAD or may indicate endothelial dysfunction or net activation of the fibrinolytic system due to underlying atherosclerosis [88]. Baseline venous plasma t-PA does not predict local fibrinolytic capacity [16], and it is likely that it is the local t-PA release capacity that is essential for the protection against occlusive thrombus formation.

**Histone deacetylase inhibitors**

Epigenetic regulation refers to heritable and reversible changes in gene expression that are mediated by chromatin-based mechanisms and does not involve traditional gene regulation such as protein binding to enhancer or promoter regions [103,104,105,106]. The chromosomal DNA is wrapped around histones and packed into nucleosomes that build the chromatin structure [107]. The degree of histone acetylation is regulated by histone acetyltransferases (HAT), and histone deacetylases (HDAC) by adding or removing acetyl groups from histones, respectively [107]. Altering the acetylation status of histones is one of the major epigenetic mechanisms [106,108]. HDAC inhibitors are a class of chemical compounds that inhibit HDAC, which increase the degree of histone acetylation, whereby the chromatin gets a more relaxed configuration and becomes accessible to transcription factors, which leads to enhanced gene transcription.

Recent research by our group and others has shown that the t-PA-gene is very sensitive to epigenetic control mechanisms, and several HDAC inhibitors markedly upregulate t-PA-gene expression in vitro [73,74,75]. Amongst all the identified and developed HDAC inhibitors, valproic acid (VPA) is clinically well-established as one of the most commonly used antiepileptic drugs worldwide [109]. It is of great clinical importance to establish if HDAC inhibitors could be used in man to modulate the endogenous fibrinolytic system. This hypothesis is supported by pharmacoepidemiological studies, in which VPA in contrast to other antiepileptic drugs was found to significantly diminish the risk of myocardial infarction in patients with epilepsy compared with controls [110,111].

Epigenetic regulation is a rapidly growing research field. There are reports on its importance in a variety of different diseases such as CVD, inflammation, metabolic syndrome, autoimmune diseases, infections, and cancer [104,108,112]. However, knowledge about the relevance of epigenetics to the development and prevention of CVD is still very limited. Modulating gene regulation through epigenetic mechanisms may have an important clinical relevance in treating CVD in the future.
AIMS

The overall hypothesis of this project is that impaired endothelium-dependent vasodilation and impaired endogenous fibrinolytic function contribute to the increased risk for cardiovascular events in hypertensive individuals. The specific aims in Study I-IV were to investigate:

- Acute vascular effects of atorvastatin in hypertensive men.
- Acute vascular effects of sodium nitroprusside in hypertensive men.
- Valproic acid’s histone deacetylase inhibitory effect on the endogenous fibrinolytic system in man.
- Prognostic impact of hypertension on long-term outcome after percutaneous coronary intervention.
METHODS

Subjects

In Study I, 13 non-smoking male subjects with mild to moderate hypertension were included after recruitment from newspaper advertisements. All patients were on antihypertensive medication and the treatment was withdrawn at least 4 weeks prior to the study. All subjects were statin-naïve prior to the study entry.

In Study II, 12 subjects (11 men and 1 woman) with primary hypertension, treated or untreated, were recruited by advertisements or in collaboration with researchers conducting a population-screening study. Patients with overt cardiovascular disease other than hypertension, blood lipid derangement or impaired glucose tolerance were not included. Any antihypertensive medication was withdrawn at least 4 weeks prior to the study.

In Study III, 10 healthy, non-smoking male subjects, aged 50-70 years were recruited by advertisement in a local newspaper. Patients with overt cardiovascular disease, hypertension, blood lipid derangement or diabetes mellitus were not included.

In Study IV, a large register study using the Swedish Coronary Angiography and Angioplasty Register (SCAAR), data were analyzed for 175,892 patients.

The study protocols in Studies I-III were approved by the Ethics Committee of the University of Gothenburg and Study III was also approved by the Medical Products Agency in Sweden. The trials were conducted according to the Declaration of Helsinki. The nature, purpose and potential risks of the studies were carefully explained to each subject before written informed consent was obtained.

Study design and experimental protocols

Study I was an open study in which 13 hypertensive men underwent assessment of EDV, EIDV, and vasoconstriction responses of the forearm by using venous occlusion plethysmography and intra-arterial infusion of Acetylcholine, sodium nitroprusside, and Angiotensin II. The protocol was repeated 1 hour after 80 mg oral atorvastatin.

Study II was an open study where 12 hypertensive subjects underwent assessment of the capacity for acute t-PA release and EDV in the perfused-forearm model using intra-arterial Substance P infusion. During one study day the procedure was performed twice in each subject, first during untreated high blood pressure and secondly during acute blood pressure lowering with intravenous sodium nitroprusside infusion. The blood pressure reduction was aiming at intra-arterial systolic pressure ≤120 mmHg or 25% reduction in mean arterial pressure for 30 min before the second provocation.

Study III was an open study with a cross-over design in which 10 healthy men were treated with valproic acid (VPA; Ergenyl Retard) 500 mg depot tablets twice daily for 2 weeks. The capacity for stimulated t-PA release was assessed in the perfused-
forearm model using intra-arterial Substance P infusion and venous occlusion plethysmography. Each subject was investigated twice, untreated and after VPA treatment, with 5 weeks wash-out in-between. The subjects were allocated to group A (n=5) or B (n=5). Group A received Ergenyl Retard for two weeks before the first examination while group B received Ergenyl Retard two weeks before the second examination.

Study IV was a register study using Swedish Coronary Angiography and Angioplasty Register (SCAAR) where data were analyzed for all consecutive patients who underwent percutaneous coronary intervention (PCI) due to ST-elevation myocardial infarction (STEMI), non-ST-elevation myocardial infarction (NSTEMI)/unstable angina (UA) or stable angina pectoris in Sweden between January 1995 and May 2013. A total of 175,892 patients were included in the analysis. We compared patients with and without hypertension. The primary outcome was all-cause mortality at any time during the study period. Subgroup analyses were performed by inclusion of interaction terms between presence of hypertension and gender, diabetes mellitus, smoking or PCI indication, i.e. STEMI, NSTEMI/UA, or stable angina pectoris. All subgroup analyses were performed in risk factor-adjusted models.

An overview of the studies is shown in Table 1.

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<td>Intervention</td>
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<td>Primary outcome</td>
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**Venous occlusion plethysmography**

In Studies I-III, forearm plethysmography was used to assess FBF, a state-of-the-art method for in vivo vascular function assessment [113,114]. A mercury-in-silastic strain gauge, connected to the calibrated plethysmograph, was placed around the widest part of the forearm to record the increase in circumference of the forearm during venous occlusion. Venous occlusion was achieved by rapid inflation of a blood pressure cuff on the upper arm to 40-50 mmHg using MAPPC® software (Elektromedicin AB) (Studies I-III). FBF was calculated and expressed as ml/min and liter of tissue.
Means of 3 to 5 recordings were calculated at each time point of measure. Average intra-observer and inter-observer coefficients of variation in our laboratory have previously been reported to be, 5.6 and 4.6%, respectively [37]. In Study I, FBF was measured at baseline and during the last minute of each dose-step for each infusion. In Study II and III, FBF measurements were done immediately after every venous blood sampling. Forearm vascular resistance (FVR) was calculated as the ratio of MAP to FBF, and expressed in arbitrary resistance units.

**The perfused-forearm model**

This model enables the study of vascular and endothelial function in vivo in humans without systemic interference and is well suited for studies on local release rates of fibrinolytic proteins. The principle of the model is to assess the local release in the forearm vasculature by comparing the plasma concentration of a substance in simultaneously collected arterial and venous blood, and to correct the arteriovenous gradient for the current plasma flow in the forearm [11,115].

**Catheterization procedure**

An arterial cannula was inserted into the brachial artery in the non-dominant arm for drug infusions in Studies I-III.

Under sterile conditions an 18-gauge arterial polyethylene catheter (Hydrocath Arterial Catheter, Becton-Dickinson) was introduced percutaneously with the Seldinger technique [116]. The catheter was advanced approximately 10 cm in the proximal direction and connected to a 5-way stop-tap for arterial infusions, blood sampling, and blood pressure monitoring. An intravenous cannula was placed retrogradely into a deep antecubital vein in the same arm for venous blood sampling (Figure 2).

In Study II, another intravenous cannula was inserted in the other arm for infusion of nitroprusside or saline. Intra-arterial blood pressure (IABP) was recorded by an electrical transducer connected to a monitor. All experiments were performed in a temperature-regulated room. Unnecessary communication and disturbance were avoided during the experiments. After all catheters were in place, at least 30 min were allowed before starting the experiment with baseline recordings. When the experiment was completed the catheters were removed and at least 20 min of manual compression over the arterial insertion site was performed.

**Intra-arterial infusion of vasoactive substances**

In Study I, three different vasoactive substances were infused intra-arterially to assess EDV, EIDV, and vasoconstriction response. Acetylcholine (ACH) 7.5 μg/ml (1, 2, and 3 ml/min), sodium nitroprusside (SNP) 0.8 μg/ml (1, 2, and 3 ml/min), and Angiotensin II (Ang II) 0.5 μg/ml (0.25, 0.5, 1, and 2 ml/min) were infused in the given order at increasing rates. Drug infusions were given at a constant infusion rate over a period of 5 min for each dose, with 5 min wash-out period between the different drugs. The subjects were monitored with ECG during the whole experiment.
Figure 2. The perfused forearm model. The arterial catheter (for arterial t-PA sampling, infusion of vasoactive drugs, and IABP measurements) and a venous cannula (for venous t-PA sampling) were placed in the antecubital fossa. The mercury-in-silastic strain gauge, used to measure forearm circumference was positioned right below, and the inflating cuff interrupting venous drainage was placed around the upper arm. The photo is taken from one of study patients.

In Studies II and III, stimulated release rate of t-PA was assessed during intra-arterial infusion of Substance P (Substance P; Clinalfa). Substance P was dissolved in saline to a concentration of 8 pmol/ml and infused into the brachial artery at a constant rate of 1 ml/min for 20 min. Post-infusion recordings were performed for 15-20 min. The dose of Substance P was chosen to obtain maximal t-PA release without systemic effects.

**Blood sampling**

In Study I, no blood sampling was performed during the experiments.

In Studies II and III, blood sampling was performed according to a strict protocol during the experiments. During pre- and post-infusion 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 1/10 vol. 0.45 M sodium citrate buffer, pH 4.3 (Stabilyte®; Biopool AB) for determination of fibrinolytic proteins. Tubes were kept on ice until plasma was isolated by centrifugation at 4°C and 2000 g for 20 min. Plasma aliquots were immediately frozen and stored at −70°C until assay. Arterial hematocrit was determined in duplicate using micro-hematocrit centrifuge.
Calculation of t-PA release

Net release or uptake of t-PA at every blood sampling point was calculated according to the formula:

\[
\text{Net release} = (C_V - C_A) \times \text{FPF}
\]

\(C_V\) is venous concentration of t-PA and \(C_A\) is arterial concentration of t-PA [11,115]. FPF was calculated from FBF and arterial hematocrit, and corrected for 1% trapped plasma according to the formula: 
\[
\text{FPF} = \text{FBF} \times \frac{(101 - \text{hematocrit})}{100}
\]

The accumulated t-PA release was calculated as area under the curve from start of the substance P infusion until the last post-infusion measurement.

Biochemical assays

Plasma concentrations of total t-PA antigen and PAI-1 antigen were determined by enzyme-linked immunosorbent assays (TintElize t-PA, Triolab; Coalize PAI-1, Chromogenix AB) according to the manufacturer’s protocol. Both assays detect free and complexed forms of the respective proteins with equal efficiency, according to the product sheets. Samples from one experiment were assayed in duplicate on the same microtest plate.

Platelet function in Study III was analyzed using multiple electrode aggregometry according to manufacturer protocol (Multiplate; Verum Diagnostica GmbH, Germany). The platelet function was analyzed with adenosine-diphosphate test (ADP-test), arachidonic acid test (ASPI-test), thrombinreceptor peptide (TRAP-test) and ristocetin-test (RISTO-test).

Blood chemistry analyses were performed by standard methods at the Department of Clinical Chemistry at the Sahlgrenska University Hospital, Gothenburg, Sweden.

Statistics

Unless otherwise stated, values are presented as mean and standard error of the mean (SEM). Paired-samples t-test was used when appropriate. Responses to vasoactive substances were analyzed using two-way ANOVA (treatment/no treatment and dose or treatment/no treatment and time). A one-way ANOVA was used for analysis of repeated measurements. Statistical analyses were performed with SPSS (version 15 and 18.0, SPSS, Chicago, Illinois)(Study I-III), Prism 3.0 (GraphPad Incorporated) (Study II), and Stata® software (version 13.1, StataSorp, College Station, Texas, USA)(Study IV).

In Study IV, we fitted unadjusted, age- and gender-adjusted, and risk factor adjusted Cox proportional hazards models on complete case data as well as on imputed data. Findings were considered significant at \(p<0.05\) (two tailed tests).
RESULTS

Study I

ATV treatment significantly increased baseline FBF from 3.38 (0.27) to 4.31 (0.35) ml/min/100 ml tissue (p<0.05, ANOVA) whereas other hemodynamic parameters such as blood pressure and heart rate were unchanged (Table 2).

Table 2. Baseline hemodynamic variables

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before ATV</th>
<th>After ATV</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure, mmHg*</td>
<td>165.1 (4.0)</td>
<td>169.0 (4.5)</td>
<td>ns</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg*</td>
<td>84.9 (1.9)</td>
<td>86.5 (2.0)</td>
<td>ns</td>
</tr>
<tr>
<td>Mean arterial blood pressure, mmHg*</td>
<td>112.7 (2.2)</td>
<td>115.6 (2.3)</td>
<td>ns</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>59 (2)</td>
<td>61 (2)</td>
<td>ns</td>
</tr>
<tr>
<td>Forearm blood flow, mL/min/100 ml tissue**</td>
<td>3.38 (0.27)</td>
<td>4.31 (0.35)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*Blood pressure measured intra-arterially before the experiments. ** Measured and calculated before the different infusions.

Endothelium-dependent vasodilation

Intra-brachial ACH infusion increased FBF in a dose-dependent manner during both experiments (p<0.001, ANOVA). ATV induced an upward shift of the dose-response curve (p<0.05, ANOVA) but did not affect the EDV per se (p=ns, two-way ANOVA, Figure 3). ACH infusion resulted in a dose-dependent decrease in FVR (p = 0.0002, ANOVA), from 4.7 (0.6) to 1.4 (0.4) arbitrary units (AU), and from 4.1 (0.5) to 1.2 (0.3) AU, before and after ATV treatment, respectively (p=ns, two-way ANOVA). Ischemia-induced reactive hyperemia resulted in a substantial increase in the FBF, from 2.8 (0.5) to 31.9 (1.2) ml/min/100 ml tissue, and from 3.5 (0.6) to 32.6 (2.6) ml/min/100 ml tissue, before and after ATV treatment, respectively (p=ns, t-test).

Endothelium-independent vasodilation

Intra-brachial SNP infusion resulted in a significant and dose-dependent increase in FBF (p<0.01, ANOVA), and ATV treatment induced an upward shift in the dose-response curve (p<0.05, ANOVA, Figure 4). In parallel, FVR decreased (p=0.0001, ANOVA), from 3.7 (0.6) to 1.6 (0.1) AU, and from 2.7 (0.3) to 1.5 (0.2) AU, before and after ATV treatment, respectively (p<0.05, two-way ANOVA).
Figure 3. Endothelium-dependent vasodilation. Forearm blood flow during baseline and in response to intra-arterial infusion of acetylcholine, before atorvastatin (■) and after atorvastatin (○) treatment. *P<0.05 (ANOVA for treatment), **P=ns (2-way ANOVA, treatment x dose). Mean and SEM.

Figure 4. Endothelium-independent vasodilation. Forearm blood flow during baseline and in response to intra-arterial infusion of nitroprusside, before atorvastatin (■) and after atorvastatin (○) treatment. *P<0.05 (ANOVA for treatment), **P=ns (2-way ANOVA, treatment x dose). Mean and SEM.

Angiotensin II mediated vasoconstriction

Intra-brachial infusion of Ang II induced a dose-dependent decrease in FBF (p<0.001, ANOVA). This vasoconstrictor response to Ang II was diminished by ATV treatment (p=0.005, two-way ANOVA, Figure 5). FVR increased during Ang II infusion and the response was inhibited by ATV (Figure 6).
Figure 5. Vasoconstriction. Forearm blood flow during baseline and in response to intra-arterial infusion of angiotensin II, before atorvastatin (■) and after atorvastatin (○) treatment. *P=0.005 (2-way ANOVA, treatment x dose). Mean and SEM.

Figure 6. Forearm vascular resistance during baseline and in response to intra-arterial infusion of Angiotensin II, before atorvastatin (■) and after atorvastatin (○) treatment. *P<0.05 (T-test at dose 0.5 μg/min), **P=ns (2-way ANOVA, treatment x dose). Mean and SEM. AU=arbitrary units.

Study II

Hemodynamic responses

Baseline hemodynamic and fibrinolytic variables before the two infusions are shown in Table 3. The blood pressure was significantly lowered by SNP infusion with baseline MAP 108.9 (3.9) mmHg and 82.4 (3.9) mmHg, during high-pressure condition
(without SNP), and low-pressure condition (with SNP), respectively (p<0.001, t-test). This resulted in 23% lower MAP on the average, during low-pressure conditions (p<0.001, t-test). Baseline FBF and FVR were similar during high- and low-pressure conditions (p=ns, t-test, Table 3). Intra-brachial infusion of substance P resulted in a significant increase in FBF at all occasions (p<0.001, ANOVA). FVR and FBF responses to substance P infusion were similar during high- and low-pressure conditions (p=ns for both, 2-way ANOVA, Figure 7).

### Table 3. Baseline hemodynamic and fibrinolytic variables

<table>
<thead>
<tr>
<th>Parameter</th>
<th>High pressure</th>
<th>Low pressure</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure, mmHg*</td>
<td>151.7 (3.0)</td>
<td>117.9 (3.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg*</td>
<td>80.7 (2.5)</td>
<td>64.8 (2.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean arterial blood pressure, mmHg*</td>
<td>108.6 (2.6)</td>
<td>83.0 (2.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Forearm blood flow, mL/L tissue</td>
<td>36.7 (3.1)</td>
<td>33.7 (2.7)</td>
<td>ns</td>
</tr>
<tr>
<td>FVR, arbitrary units</td>
<td>3.29 (0.26)</td>
<td>2.85 (0.31)</td>
<td>ns</td>
</tr>
<tr>
<td>Plasma t-PA antigen, ng/mL</td>
<td>9.05 (0.40)</td>
<td>8.96 (0.52)</td>
<td>ns</td>
</tr>
<tr>
<td>t-PA release, ng/min/L tissue</td>
<td>12.4 (4.0)</td>
<td>15.8 (5.7)</td>
<td>ns</td>
</tr>
</tbody>
</table>

*Blood pressure measured intra-arterially before the experiments. Abbreviations: FVR=forearm vascular resistance, t-PA=tissue plasminogen activator

![Figure 7](image-url)  
**Figure 7.** Forearm vascular resistance (arbitrary units) during baseline and in response to 20 minutes of intra-arterial infusion of substance P (8 pmol/min). During High pressure condition (without SNP infusion) (○) and during Low pressure condition (with SNP infusion) (■). Baseline measurements 15 minutes before and 20 minutes after the infusion. 2-way ANOVA, mean and SEM.
t-PA release responses
Substance P induced a significant increase in t-PA release from the forearm both during high and acutely lowered blood pressure (p<0.01 for both, ANOVA). The t-PA antigen release response to intra-brachial substance P infusion was similar during high- and low-pressure conditions (p=ns, 2-way ANOVA, Figure 8). The peak t-PA release rate was 199 (77) ng/min/L and 167 (41) ng/min/L tissue during high- and low-pressure conditions, respectively (p=ns, t-test). Accumulated t-PA release, measured as area-under-the-curve, was almost identical, 2.395 (750) and 2.394 (473) ng/L tissue, during high- and low-pressure conditions, respectively (p=ns, t-test). The average time to peak t-PA secretion was 5.4 (1.9) and 5.8 (1.7) minutes during high- and low-pressure conditions, respectively (p=ns, t-test).

Reference group
In the three reference subjects that were investigated without blood pressure reduction, hemodynamic and t-PA responses were similar during both stimulations (p=ns, 2-way ANOVA; data not shown).

Study III
Hemodynamic responses
Baseline hemodynamic and fibrinolytic variables before the two infusion studies are shown in Table 4. Baseline FBF was similar in VPA treated and untreated patients (p=ns, t-test, Table 4). Systolic and diastolic blood pressure levels were unaffected by VPA treatment (p=ns, t-test). Intra-brachial Substance P infusion resulted in a signifi-
cant increase in FBF on both occasions (p<0.0001 for both, ANOVA). FBF responses to Substance P infusion showed a tendency to suppression after VPA treatment, compared to untreated subjects (p=0.057, 2-way ANOVA, Figure 9).

Table 4. Study parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before VPA</th>
<th>After VPA</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum valproate, μmol/L</td>
<td>0</td>
<td>426 (25)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Baseline FBF, ml/min/100 ml tissue</td>
<td>2.84 (0.19)</td>
<td>2.73 (0.18)</td>
<td>ns</td>
</tr>
<tr>
<td>Baseline venous t-PA antigen, ng/ml</td>
<td>10.54 (0.65)</td>
<td>8.57 (0.41)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Baseline t-PA release, ng/min/L tissue</td>
<td>2.16 (3.3)</td>
<td>3.27 (1.4)</td>
<td>ns</td>
</tr>
<tr>
<td>Baseline venous PAI-1 antigen, ng/ml</td>
<td>22.2 (4.6)</td>
<td>10.8 (2.1)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Baseline venous t-PA:PAI-1 ratio</td>
<td>0.74 (0.17)</td>
<td>1.03 (0.17)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Baseline arterial t-PA:PAI-1 ratio</td>
<td>0.85 (0.17)</td>
<td>1.19 (0.20)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Figure 9. Forearm blood flow during baseline and in response to 20 min infusion of Substance P (8 pmol/min) in untreated (■) and VPA treated (○) healthy subjects (n=10). Baseline measurements 15 min before and 10 min after the infusion. 2-way ANOVA, mean and SEM.
**t-PA baseline levels and responses**

VPA decreased baseline venous t-PA antigen levels by approximately 20% (p<0.05, t-test, Table 4). In 9 of 10 patients baseline t-PA antigen decreased after VPA treatment (p=0.02, and p=0.01, Binomial Test and Related-Samples Wilcoxon Signed Rank Test, respectively). Baseline t-PA release was 2.16 (3.3) and 3.27 (1.4) ng/min/L tissue during untreated and VPA treated conditions, respectively (p=ns, t-test). t-PA Antigen release in response to intra-brachial Substance P infusion was not affected by VPA treatment (p=ns, 2-way ANOVA, Figure 10). The average time to peak t-PA secretion in untreated and treated subjects was 13.2 (1.7) and 11.0 (2.1) minutes, respectively (p=ns, t-test).

**Figure 10.** Net forearm release rates of t-PA antigen during baseline and in response to 20 min infusion of Substance P (8 pmol/min) in untreated (■) and VPA treated (○) healthy subjects (n=10). Baseline measurements 15 min before and 10 min after the infusion. 2-way ANOVA, mean and SEM.

**Plasminogen activator inhibitor-1 levels**

Circulating plasma levels of PAI-1 were significantly reduced from 22.2 (4.6) to 10.8 (2.1) ng/ml after VPA treatment (p<0.05, t-test, Figure 11). In all 10 patients baseline venous PAI-1 antigen decreased after VPA treatment (p=0.002, and p=0.005 for binomial Test and Related-Samples Wilcoxon Signed Rank Test, respectively). During substance P infusion, there was no detectable release of PAI-1 from the forearm, either in the treated or untreated condition (p=ns, data not shown).
**Figure 11.** Baseline venous and arterial plasminogen activator inhibitor-1 (PAI-1) antigen levels in untreated (●) and VPA treated (grey square) subjects. *p<0.05, paired t-test, untreated versus treated.

### Fibrinolytic balance

The ratios of baseline venous t-PA:PAI-1, and baseline arterial t-PA:PAI-1 antigens were both significantly increased by VPA treatment (p<0.01 for both, t-test, Table 4).

### Platelet count and aggregation tests

VPA treatment did not alter platelet counts (p=ns, t-test). Platelet aggregation responses were evaluated in whole blood samples using Multiplate analysis induced by ASPI, ADP, TRAP, and RISTO-low and -high, respectively. Platelet aggregation was not affected by VPA-treatment (p=ns, t-test, data not shown).

### Study IV

Between January 1995 and May 2013, a total of 175,892 patients underwent PCI in Sweden for the indications of STEMI, NSTEMI/UA or stable angina. For 16,451 patients (9%) data was missing regarding hypertension. Baseline characteristics of the hypertensive and non-hypertensive groups are shown in Table 5. Hypertensive patients were older and 33% were women compared to 25% in the non-hypertensive group. Diabetes mellitus, hyperlipidemia, previous myocardial infarction (MI), previous PCI, and previous coronary artery bypass grafting (CABG) were all more common in the hypertensive group. One vessel disease was more common in non-hypertensive patients, while two vessel, three vessel and left main disease were more common among hypertensive patients. STEMI was more common in non-hypertensive patients, while NSTEMI/UA and stable angina were more frequent in the hypertensive group (Table 5).
Table 5. Patient characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>HT</th>
<th>No HT</th>
<th>Missing N (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD)</td>
<td>68 ± 10</td>
<td>64 ± 11</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Women, n (%)</td>
<td>25.788 (33)</td>
<td>20.202 (25)</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoker, n (%)</td>
<td>12.541 (17)</td>
<td>21.052 (28)</td>
<td>24.551 (14)</td>
<td>0.532</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>19.583 (25)</td>
<td>8.263 (10)</td>
<td>14.639 (8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>46.994 (62)</td>
<td>24.887 (32)</td>
<td>20.334 (12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Previous MI, n (%)</td>
<td>19.787 (26)</td>
<td>15.268 (19)</td>
<td>6.212 (4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Previous PCI, n (%)</td>
<td>3.407 (4)</td>
<td>2.304 (3)</td>
<td>1.902 (1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Previous CABG, n (%)</td>
<td>8.705 (11)</td>
<td>4.470 (6)</td>
<td>2.512 (1)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Findings on CA

| Normal/atheroma | 1.900 (3) | 1.520 (2) | -            | <0.001  |
| One vessel      | 29.229 (41) | 36.534 (52) | -            |         |
| Two vessel      | 21.461 (30) | 19.739 (28) | -            |         |
| Three vessel    | 14.740 (21) | 10.186 (14) | -            |         |
| Left main disease | 4.192 (6) | 2.524 (4) | -            |         |

Indication for CA

| STEMI            | 16.057 (21) | 25.891 (32) | -            |         |
| NSTEMI/UA        | 39.772 (51) | 39.072 (48) | -            |         |
| Stable angina    | 22.271 (29) | 16.378 (20) | -            |         |

Abbreviations: CA=coronary angiography; CABG=coronary artery bypass grafting; MI=myocardial infarction; NSTEMI=non-ST-elevation myocardial infarction; PCI=percutaneous coronary intervention; SD= standard deviation; STEMI=ST-elevation myocardial infarction; UA=unstable angina.

Primary analysis

Unadjusted long-term survival was lower in hypertensive patients (Figure 12 and 13). First-year mortality was 5.3% and 4.1% in the hypertensive and non-hypertensive group, respectively, whereas the corresponding total mortality at any time during the study period was 20.4% and 17.6%, respectively. Hypertension was associated with increased long-term risk of death both in unadjusted and adjusted models. Hypertension significantly increased HR for overall mortality in unadjusted, age and gender matched and risk factor adjusted complete case analysis. This finding was consistent in complete case analyses as well as in analyses on imputed data sets (Figure 14). The median follow-up time was 1862 days for patients with data for hypertension, and 1636 days for patients with a diagnosis of hypertension.

Subgroup analyses

Hypertension had the greatest impact on mortality for patients with STEMI and the least for patients with stable angina pectoris (p=0.001, Cox model interaction term) (Figure 15). The presence of hypertension also influenced prognosis to a greater degree among smokers than non-smokers (p=0.002), and among young patients compared to older patients (p<0.001). We found no significant interaction between hypertension and gender or diabetes mellitus (Figure 15).
Figure 12. Unadjusted Kaplan-Meier survival curve for patients with hypertensive diagnosis and non-hypertensive diagnosis with ischemic heart disease that undergo PCI.

Figure 13. Cumulative hazards ratio estimate for patients with ischemic heart disease with hypertension diagnosis and no-hypertension diagnosis that undergo PCI, respectively.
Figure 14. Hazard ratios (HR) for overall mortality. Hypertension significantly increased HR for overall mortality in unadjusted, age and gender matched, and risk factor adjusted analysis (p<0.001 for all) in complete case analyses as well as in analyses on imputed data sets. MI=multiple imputation.

Figure 15. Hazard ratios (HR) for interactions with hypertension. Age group (p<0.001) and smoking status (p=0.002) had significant interactions with hypertension. Diabetes mellitus and gender did not have any significant interactions (p=ns for both). PCI indication (NSTEMI/UA, stable CAD, and STEMI) had significant interaction (p=0.0005) with hypertension.
DISCUSSION

Hypertension is a major risk factor for cardiovascular disease and has a substantial impact on the burden of cardiovascular morbidity and mortality worldwide. Nevertheless, it is not fully understood which pathophysiological factors underlie the increased risk of atherothrombotic events in hypertensive patients and how to decrease the risk in these patients except by lowering the blood pressure levels.

This thesis is based on acute pharmacological interventional studies of endothelial function in man and a long-term register study to investigate the impact of hypertension after PCI. Acute pharmacological actions on endothelial and vascular function of different drugs (ATV, Nitroprusside, VPA; Study I-III) were studied in the invasive studies. We tried to clarify and intervene with the mechanisms behind the impaired endothelial function both with regard to impaired endogenous fibrinolysis and EDV in hypertensive subjects. Furthermore, we investigated the epigenetic regulation of the endogenous fibrinolytic system in healthy man (Study III). A large-scale register study on the prognostic role of hypertension on long-term survival after PCI was performed since the literature is limited and inconsistent (Study IV).

Acute vascular effects of atorvastatin in hypertension

The study demonstrated that a single dose of ATV affects peripheral vascular reactivity in hypertensive patients. Basal blood flow, EIV, and Ang II-induced vasoconstriction were all significantly modified by ATV, whereas endothelium-dependent vasodilator responses were unchanged. Potential explanations for our findings include different vasoprotective effects of statins that are independent of their cholesterol-lowering actions, commonly named pleiotropic effects [50,117]. Such pleiotropic effects include improvement of endothelial function via reduction of oxidative stress [118], or on rapid activation of endothelial nitric oxide synthase [118,119]. Statins may induce acute vasodilation through both endothelium-dependent and endothelium-independent mechanisms in vitro, where the latter depends on vascular smooth muscle cell (VSMC) hyperpolarization [120]. The observed increase in basal blood flow in the forearm after ATV treatment is in line with results from a previous report in which a single dose of ATV significantly increased baseline diastolic blood flow in the coronary circulation [121]. Rapid improvement of endothelial function has previously been demonstrated in non-invasive studies within 24 h after initiation of atorvastatin treatment in healthy subjects and in smokers [122,123].

In our study, endothelium-dependent vascular responses were unaffected by ATV. Whether the lack of effect could be related to the hypertensive state of the subjects or to other methodological differences of the studies remains unknown. Statins are able to induce acute vasorelaxant endothelium-independent effects on aortic rings from hypertensive rats, and it has been suggested that the effects on VSMC involve blocking of extracellular calcium entry [124]. In vitro, statins can acutely inhibit vasoconstriction by endothelium-independent mechanisms in different pathways involving Ang II [124,125]. We could demonstrate that endothelium-independent vascular responses in
terms of NO-induced vasodilation, and Ang II-induced vasoconstriction, were both modified by ATV. The results suggest that acute statin effects in hypertension are independent of the endothelium, indicating that vascular smooth muscle cells are affected more rapidly than the endothelial cells by ATV in vivo. These actions may in part contribute to the beneficial pleiotropic effects of statins even in the acute in vivo setting.

Antihypertensive therapy and t-PA release

Importance of functional t-PA release

During thrombus formation in vivo, a rapid onset of a regulated t-PA release response is probably pivotal for limiting clot expansion, since t-PA is two orders of magnitude more effective when present during clot formation than in dissolving an existing thrombus [80,81]. An acute release of t-PA can be initiated within less than a minute in healthy man and the release increases manifold with stimulation [11,12,85,126]. The t-PA response is impaired in hypertensive patients [6,92], and in CAD [7]. Our research group has previously shown that chronic antihypertensive therapy restores the impaired t-PA release in hypertension [37].

Effect of acute blood pressure lowering

To further explore the mechanisms behind the regulatory effects of high blood pressure on t-PA release, we tested if t-PA release is affected by acute blood pressure lowering. We found stimulated t-PA release responses to reach similar low levels in untreated hypertensive patients as reported recently [37]. However, in contrast to chronic blood pressure treatment the stimulated t-PA release capacity was unaffected by acute lowering of blood pressure to near-normal levels by the endothelium-independent NO-donor nitroprusside in this study. The results clearly suggest that blood pressure needs to be sustained at a normal level for a longer time before the capacity for t-PA release is restored. It is likely that the main cause of the impaired fibrinolysis in hypertension is a decrease in the endothelial pool of t-PA which normally is ready to be released upon stimulation. The rationale for investigating the effect of acute blood pressure lowering was the findings in a previous ex vivo study using intact vessels indicating that high intraluminal pressure suppresses t-PA release into the perfusion medium within 1-2 h, suggesting receptor-related protein release mechanisms rather than transcriptional regulation to be involved [31]. Both constitutive and regulated releases of t-PA from the endothelium are dependent on increases in intracellular calcium levels [127,128]. It has been demonstrated that acute administration of 17 b-oestradiol locally in the forearm augmented bradykinin induced t-PA release [129]. The authors postulated the effect to be mediated through activation of an endothelial cell-surface oestrogen receptor pathway that has been shown to rapidly increase intracellular calcium concentrations [130]. In another study, acute administration of ascorbic acid was found to improve endothelial t-PA release in heavy smokers, suggesting the mechanism of recovery to be conducted through reduction in oxidative stress [131]. Thus, acute modulators of stimulated t-PA release exist, and we hypothesized that decreased mechanical forces on endothelial cells in hypertension could be one of them. We found the capacity of t-PA release, as well as EDV responses, to be unaffected by acute blood pressure lowering. It is unlikely that a larger sample size could lead to
positive results as the t-PA responses during high and low blood pressure were almost identical. Our neutral results support the hypothesis that the impaired t-PA release in hypertension is explained by a decreased cellular content of t-PA.

**Profibrinolytic effect of HDAC inhibitors in vivo**

Pharmacological means to directly target and improve the endogenous fibrinolytic system have been lacking. Therefore, we tried to translate very promising findings in the emerging field of epigenetic cardiovascular research, in which recent work by our group has shown that the t-PA gene is very sensitive to epigenetic control mechanisms, and several HDAC inhibitors including VPA markedly up-regulate the t-PA gene expression in vitro. We performed a proof-of-concept study investigating if VPA can affect the fibrinolytic system in normotensive healthy men. Our main finding was a markedly reduced level of circulating plasma PAI-1 antigen by 51% after VPA treatment. This indicates that the fibrinolytic balance between t-PA and PAI-1 antigen level was altered, which may result in a profibrinolytic state in man. The capacity for acute stimulated t-PA antigen release was not affected by VPA treatment.

There are several potential explanations why the t-PA release capacity was not improved by VPA. Most importantly, healthy subjects in this study might have a normal t-PA production and release capacity from the beginning, and VPA would not be expected to increase the intracellular t-PA pool to supra-physiological levels. In a recent study using a porcine ischemia model, VPA has showed to increase coronary t-PA release [132]. Therefore, after completing the VPA study in healthy subjects, our group went further and performed a related clinical study in patients with atherosclerosis, where the t-PA release capacity is predicted to be impaired from the beginning. The study demonstrated that VPA augmented cumulative t-PA release capacity during repeated stimulation sequences in post-myocardial infarction subjects [133]. Importantly, the previous finding of diminished PAI-1 after VPA treatment in Study III was also confirmed in patients with CAD. Some methodological differences between the studies should be noted. In the study of atherosclerotic men, several new methodological aspects were implemented. Repeated stimulated t-PA release was used instead of a single stimulation of t-PA release. The effect of VPA on t-PA release capacity was only observed after the repeated stimulation sequence, measured as reduction in the exhaustion of cumulative t-PA release during the second stimulation [133]. Furthermore, isoprenaline was used instead of substance P to stimulate the t-PA release. It cannot be excluded that isoprenaline and/or repeated stimulation sequences could have led to other results even in Study III.

Our finding of diminished circulating levels of PAI-1 antigen after VPA treatment is very interesting and might translate into a decreased cardiovascular risk, as PAI-1 is the main inhibitor of active t-PA, and thereby of vascular fibrinolysis [134]. A well-known side effect of VPA is thrombocytopenia but we did not observe any significant suppression of platelet count or function after 2 weeks of VPA treatment. Thus, the diminished PAI-1 after VPA cannot be explained by reduced platelet number which would have been an explanation otherwise as the main source of plasma PAI-1 is the platelets. In vitro, clinical doses of VPA did not have any effect on PAI-1 gene transcription in human umbilical vein endothelial cells [73].
A protective effect of VPA on cardiovascular disease is supported by results in recent pharmacoepidemiological studies, in which VPA in contrast to other antiepileptic drugs was found to significantly diminish the risk of myocardial infarction by 40% in patients with epilepsy compared with controls [110,111] in Danish register studies. A similar association was also found in a nested case-control study on the Irish population [135]. A possible explanation to the observed cardiovascular protection by VPA might be related to the diminished levels of PAI-1, and enhanced t-PA release capacity in patients with atherosclerosis, resulting in an increased fibrinolytic capacity. Further studies to investigate the mechanisms behind the down-regulatory effect of VPA on PAI-1 levels are motivated. New clinical trials with VPA or other HDAC inhibitors are also warranted to explore long-term effects on the fibrinolytic system in patients with atherosclerosis, regarding both effect size and safety in combination with platelet inhibition therapy.

**Long-term impact of hypertension after PCI**

A large-scale register study was performed to clarify the prognostic role of hypertension on long-term survival after PCI. We studied the impact of hypertension on mortality in 175,892 consecutive patients who underwent PCI in a prospective SCAAR register study. 78,100 patients had a history of hypertension. We found that hypertension was associated with increased mortality among patients with ischemic heart disease who undergo PCI, particularly among patients with STEMI. Several mechanisms may contribute to explain a potential impact of hypertension on outcome after PCI. It has been considered that ACS patients with hypertension represent a subset at higher risk profile, such as older age, and higher comorbidities [136,137,138,139,140]. We could confirm those observations as we found that hypertensive patients were older and had higher prevalence of diabetes, three vessel and left main disease, as compared to patients without hypertension. However, hypertension was an independent predictor of long-term mortality after adjustment for age, gender and traditional risk factors. To our knowledge this is the largest study so far that addresses the influence of hypertension on long-term mortality following PCI. The prevalence of hypertension was comparable to that reported in previous studies, being present in half of the population [141]. Hypertension was associated with highest risk in patients with STEMI, it had intermediate risk in patients with NSTEMI/UA while the risk was lowest in patients with stable angina. There are several possible mechanistic explanations for why hypertension may be particularly deleterious in patients after acute myocardial infarction. High blood-pressure in patients after acute MI accelerates and worsens the process of post-infarction cardiac remodelling. The key clinical features of hypertensive heart disease are development of LV hypertrophy with heart failure, propensity to develop malignant ventricular arrhythmias, acceleration of coronary atherosclerosis and chronic alteration in renin-angiotensin system. The increased hemodynamic and wall stress imposed by hypertension alters ventricular topography adversely.

Furthermore, chronic alterations in renin-angiotensin-aldosterone system with over-expression of angiotensin II receptor 1 (AT1) may induce a vicious circle inducing low grade inflammation, myocardial fibrosis, increased extracellular matrix, endothelial dysfunction, diastolic dysfunction etc, resulting in worsened prognosis after MI [142,143,144]. A mechanistic explanation for the adverse impact of hypertension in
patients with STEMI undergoing PCI may be its association with impaired reperfu-
sion [44,45]. Impaired coronary microvascular function can contribute to explain the
impaired reperfusion associated with hypertension in patients with STEMI [45].

This large-scale register study has several limitations. Unfortunately, we did not have
data on duration or severity of hypertension of the patients. Second, the recorded data
on hypertension status in our study neither distinguish treated from non-treated pa-
tients, well regulated from un-regulated blood pressure nor different antihypertensive
agents from each other as no data was available for exact blood pressure levels or an-
tihypertensive drug treatment in the register. All these factors may underestimate the
adverse impact of hypertension for long-term outcome after PCI since a proportion of
hypertensive patients probably have well-treated blood pressure prior to PCI. Third,
no data were available on prevalence of impaired left ventricular ejection fraction,
heart failure, left ventricular hypertrophy or previous stroke. Fourth, a proportion of
patients had missing data (9%) regarding hypertension status, which may have caused
biased results. A strength, however, was that the results from the multiple imputation
model were congruent with the data from the complete case analysis.

In conclusion, hypertension was associated with increased mortality risk in patients
who are revascularized with PCI. This risk was lowest in patients with stable angina
and highest in patients with STEMI.

**Concluding remarks and future perspectives**

Endothelial dysfunction, both in terms of impaired EDV and fibrinolytic capacity, is
a common denominator in patients with hypertension and manifest atherosclerosis.
Rapid improvement of endothelial dysfunction in secondary prevention settings by
different pharmacological interventions could have important clinical implications in
patients with ACS. Given the obvious adverse prognostic impact of ED there is a
clinical need to seek for new treatment options to directly target the endothelium for
reversing ED regarding both EDV and fibrinolytic capacity in both primary and sec-
ondary prevention settings.

In this thesis, three clinical experimental studies were performed in hypertensive and
normotensive subjects in order to investigate endothelial and vascular effects of sev-
eral pharmacological interventions with different pharmacodynamic actions. Effects
on the endogenous fibrinolytic system and EDV responses were studied. The endo-
thelium was exposed to pharmacological interventions in order to rapidly improve
endothelial or vascular function in man. We could not demonstrate any endothelium-
dependent effects in the forearm by any of the pharmacological stimuli we used, re-
garding EDV (Studies I-III), and stimulated t-PA release (Studies II-III). Unexpect-
edly, the pleiotropic beneficial vascular effects of atorvastatin in hypertension were
apparently endothelium-independent. Acute blood pressure lowering by nitroprusside
did not improve stimulated t-PA release in hypertension, suggesting diminished re-
leasable t-PA pool in the endothelium of hypertensive patients. Recent interesting
findings from the evolving cardiovascular research field of epigenetics have dem-
onstrated that HDAC inhibitors, including VPA markedly up-regulate the t-PA gene
transcription in vitro. In this thesis, effects of VPA on the endogenous fibrinolytic system were studied in healthy man. We found that VPA alters the fibrinolytic system in a profibrinolytic direction, in this study mainly by diminishing PAI-1 rather than by enhancing t-PA release. The long-term adverse impact of hypertension diagnosis on survival after PCI was demonstrated in a large-scale register study, and the highest risk was found in patients with STEMI. The findings underscore the importance of an optimal secondary prevention including blood pressure control in patients with CAD.

Further clinical studies investigating the long-term effects of HDAC inhibitors on the fibrinolytic system in patients with manifest atherosclerosis and hypertension are warranted. The new concept of pharmacological stimulation of the endogenous fibrinolytic system by HDAC inhibitors might have a potential to become a complement to today’s ACS treatment arsenal or as a prophylactic antithrombotic treatment in patients with residual increased thromboembolic risk despite optimal established preventive therapy.
CONCLUSIONS

The observed acute statin effects on vascular function in hypertension seem to be endothelium-independent and related to vascular smooth muscle cell function (Study I).

Acute blood pressure lowering with endothelium-independent vasodilator does not restore the impaired fibrinolytic capacity in hypertension (Study II). The data suggest that high blood pressure decreases the endothelial t-PA pool rather than interferes with release mechanisms of the protein.

Treatment with the HDAC inhibitor valproic acid lowers plasma PAI-1 antigen levels and changes the fibrinolytic balance measured as t-PA/PAI-1 ratio in a profibrinolytic direction in healthy man (Study III). The acute stimulated t-PA release capacity was not affected by VPA treatment.

Hypertension is associated with increased mortality risk in patients who are revascularized with PCI. This risk was lowest in patients with stable angina and highest in patients with STEMI (Study IV).
Kranskärllsjukdom är en av de vanligaste dödsorsakerna i världen. I de flesta fall inträffar syrebrist i hjärtat till följd av en blodpropp i ett kranskärl. Om blodproppen inte löses upp relativt snabbt resulterar syrebristen i en hjärtinfarkt. Fibrinolys innebär upplösning av blodpropp och är en av kroppens skyddsmechanismer mot utveckling av blodproppar.


Epigenetik handlar om hur olika gener aktiveras och avaktiveras. Resultat från experimentell forskning på odlade celler har visat att t-PA genen står under epigenetisk kontroll. Valproinsyra som har epigenetiska egenskaper har visats öka t-PA i både cellodling och i grisförsök.

Målet med avhandlingen var att undersöka olika mekanismer vid nedsatt endotelfunktion samt att försöka förbättra endotelets kärllvidgande och fibrinolytiska förmåga i olika läkemedelsstudier på människa. Långtidsprognosen av patienter med högt blodtryck undersöks i en registerstudie.

I de tre kliniska läkemedelsstudierna studerades kärlfunktion och fibrinolysförmåga i underarmen på människa. Långtidsprognosen av patienter med högt blodtryck efter en hjärtinfarkt som behandlats med ballongvidgning (PCI) undersöks med data från Svenska hjärtregistret (SCAAR). Alla patienter som genomgick PCI i Sverige mellan 1995-2013 på grund av hjärtinfarkt och kärlkramp (totalt 175892 patienter) inkluderas i analysen.

Akuta effekter på blodkärl av läkemedlet atorvastatin undersöks i underarmsförsök hos försökspersoner med högt blodtryck. De observerade akuta blodkärlsvidgande effekterna av atorvastatin verkade vara oberoende av endotelet och istället relaterade till muskelcellerna i kärlvägen. Akut blodtryckssänkning med läkemedlet nitroprussid kunde inte återskapa den nedsatta t-PA friisättningsförmågan hos försökspersoner med högt blodtryck. Hos friska försökspersoner påverkades inte förmågan till stimulerad t-PA friisättning av behandling med valproinsyra. Däremot resulterade behandlingen i påtagligt sänkta nivåer av plasminogen activator inhibitor-1 (PAI-1) antigen i blodet. PAI-1 är den viktigaste hämmaren av t-PA och resultaten tolkas som att tillförsel av valproinsyra ger ökad fibrinolytisk effekt hos människa. Registerstudien visade att knappt hälften av alla patienterna hade högt blodtryck. Högt blodtryck var associerat
med ökad långtidsdödlighet och denna risk var högst i patienter yngre än 65 år, hos rökare och hos patienter med omfattande hjärtinfarkter (så kallade ST-höjningsinfarkter).

Sammanfattningsvis är endotelfunktionen försämrad hos patienter med högt blodtryck och detta bidrar till att dessa patienter har ett nedsatt försvar mot blodproppar. Högt blodtryck försämrar långtidsöverlevnaden hos patienterna efter ballongvidgning av kranskärl. Akut blodtryckssänkning återställer inte den nedsatta t-PA frisättningsförmågan och detta talar för att t-PA produktionen i endotelet är minskad hos patienter med högt blodtryck genom en annan mekanism än blodtrycksförhöjningen i sig. Resultaten understryker vikten av att förutom god blodtryckskontroll hos patienter med kranskärlssjukdom, också undersöka om behandling med epigenetiska läkemedel skulle kunna stärka kroppens försvar mot blodproppar också hos de med otillräcklig effekt av dagens standardbehandling.
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