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Regulation of Vascular Endothelial t-PA Expression in Inflammation

Potential Target for Pharmacological Modulation

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"As for me, all I know is that I know nothing"

Socrates 469-399 BC

ABSTRACT

The endogenous fibrinolytic system is important for preventing occluding thrombosis and subsequent tissue infarction. The main activator of the fibrinolytic system in the vascular compartment is tissue-type plasminogen activator (t-PA). In a clotting situation, this enzyme is acutely released from storage pools in the endothelium and initiates fibrin breakdown. The endothelial release capacity of t-PA can be impaired by genetic or functional means and this impairment has been found to be associated with an increased risk for ischemic vascular disease, including myocardial infarction. Hypertension, smoking, and atherosclerosis are among the conditions associated with reduced t-PA production and release.

Another condition that could potentially be associated with reduced fibrinolysis is inflammation, but the role of inflammation in the regulation of t-PA production has not been clearly established. Thus, we investigated the effect of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) on the production of t-PA in cultured human vascular endothelial cells. We also studied which intracellular signaling mechanisms are of importance. TNF-α and IL-1β both caused a significant reduction of t-PA mRNA and protein production in endothelial cells. This effect was most evident at time points ≥24 h. Pharmacological blocking of selected intracellular signaling pathways revealed a central role of NFκB signaling in mediating the pro-inflammatory cytokine reduction of t-PA. p38 MAPK signaling was also found to be of some importance. IL-6, on the other hand, did not cause an effect on t-PA production. In fact, further experiments showed that the endothelial cell model used does not express a complete receptor for IL-6. However, a soluble form of the IL-6 receptor exists in the circulation and when present in the cultures, a suppressive role of IL-6 on t-PA production was detected.

Given the central role of t-PA in the fibrinolytic system and the fact that impaired t-PA production is associated with increased risk of atherothrombotic events, a pharmacological means to increase the production of this enzyme could be desirable. There are indications that t-PA production could be partly regulated by epigenetic mechanisms, mainly histone acetylation. We thus investigated a panel of clinically used histone deacetylase inhibitors (HDACis) to determine their effect on t-PA production *in vitro*. All HDACis tested, irrespective of chemical structural class, potently stimulated endothelial t-PA production indicating that it is indeed their ability to modulate histone acetylation that affects t-PA synthesis. This was further supported by the fact that the HDACi valproic acid affected endothelial histone acetylation status, both globally and also specifically around the t-PA transcription start site, although in initial siRNA experiments we were unable to identify which specific HDAC enzyme(s) were of importance.

In conclusion, these data suggest that t-PA production in cultured vascular endothelial cells is suppressed by prolonged exposure to the inflammatory cytokines TNF-α and IL-1β. Moreover, IL-6, in the presence of its soluble receptor, can also attenuate t-PA production. If these results hold true also *in vivo*, it could be of importance e.g. in the local environment surrounding the inflammatory atherosclerotic plaque where these cytokines are present in high concentrations and where a sufficient t-PA production could be of uttermost importance. Clinically used HDACis potently stimulate t-PA in our experimental *in vitro* model, apparently via their effects on HDAC enzymes. As such, these substances could perhaps be considered for pharmacological stimulation of the endogenous fibrinolytic system as a novel prevention strategy for ischemic vascular disease.

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

II Larsson P., Ulfhammer E., Karlsson L., Bokarewa M., Wåhlander K., Jern S. Effects of IL-1β and IL-6 on tissue-type plasminogen activator expression in vascular endothelial cells.
Thrombosis Research 2008; 123: 342-51

III Larsson P., Bergh N., Ulfhammer E., Magnusson M., Wåhlander K., Karlsson L., Jern S. Histone deacetylase inhibitors potently stimulate tissue-type plasminogen activator production in vascular endothelial cells.
In manuscript

IV Larsson P.*, Ulfhammer E.*, Magnusson M., Bergh N., Lunke S., El-Osta A., Medcalf R.L., Svensson P-A., Karlsson L., Jern S. Role of histone acetylation in the stimulatory effect of valproic acid on vascular endothelial tissue-type plasminogen activator expression.
*Both authors contributed equally to this study.
Submitted

CONTENTS

ABBREVIATIONS

Ac	Acetyl
ANOVA	Analysis of variance
AP-1	Activator protein-1
ASA	Acetylsalicylic acid
ATF	Activating transcription factor
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CRE	cAMP response element
CREB	cAMP response element (CRE) binding protein
CRP	C-reactive protein
C_T	Threshold cycle
EGM-2	Endothelial growth medium-2
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated kinase
FDP	Fibrin degradation products
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H3K9	Histone H3, lysine 9 (example)
HAEC	Human aortic endothelial cells
HAT	Histone acetyltransferase
HCAEC	Human coronary artery endothelial cells
HDAC	Histone deacetylase
HDACi(s)	Histone deacetylase inhibitor(s)
HPRT	Hypoxanthine phosphoribosyl transferase
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Inter-cellular adhesion molecule-1
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-6R	Interleukin-6 receptor
IP	Immunoprecipitation
JNK	c-jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
mRNA	Messenger RNA
NF1	Nuclear factor 1
NF-κB	Nuclear factor-κB
PAF	Platelet activating factor
PAI-1	Plasminogen activator inhibitor-1
PBA	Phenylbutyrate
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RA	Rheumatoid arthritis
RT-PCR	Reverse transcriptase polymerase chain reaction
SCFA	Short chain fatty acid
SEM	Standard error of the mean
sIL6R	Soluble IL-6 receptor
siRNA	Short interfering RNA
Sp1	Specificity protein 1
TAFI	Thrombin-activatable fibrinolysis inhibitor
TIS	Transcription initiation site
TNF-α	Tumor necrosis factor-α
t-PA	Tissue-type plasminogen activator
TSA	Trichostatin A
u-PA	Urokinase-type plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1
VPA	Valproic acid
VPM	Valpromide
vWF	Von Willebrand factor

INTRODUCTION

Historical perspective

Although man has always been aware of the clotting behavior of blood, the first actual descriptions of blood coagulation is attributed to the works of Hippocrates and Aristotle in the 4:th century B.C. They noted that blood from sacrificed animals congealed upon collection and that bleeding from wounds stopped as a "skin" formed over the emerging blood. Aristotle further noted that there were differences in the constitution of the blood between the sexes and different ages, and furthermore, that blood from different species had different coagulation abilities [1]. The modern history of coagulation research began during the 19:th century with the identification of a number of enzymes involved in what is now known as the coagulation cascade. During the same period, intravascular thrombus formation was extensively studied by many scientists, including the German pathologist Rudolf Virchow. In 1856 he suggested that thrombus formation was the consequence of three predisposing conditions: alterations in the blood vessel wall, perturbed blood flow, and abnormalities in blood constituents [2]. Even though he originally referred to venous thrombosis, these concepts can also be applied to the arterial circulation. Remarkably this theory, although in an expanded form, still remains valid today.

Likewise, spontaneous dissolution of clots in blood from living and dead persons and animals was noted early. The lytic activity of cadaveric blood was noted to vary with the cause of death of the animal. For example, it was reported that "in animals killed by lightning or electricity" or "in animals who are run very hard and killed in such a state", the blood does not clot [3]. In the late 19:th century the concept of lysis of fibrin (fibrinolysis) was proposed, even though it was not until the second half of the 20:th century that plasminogen activators, including the type stored in the endothelium, and their inhibitors were described and their function evaluated [4].

These findings, collectively, led to the development of a concept of a "hemostatic balance" between the clotting and the fibrinolytic mechanisms; tipping the scale in one direction leading to bleeding and in the other to thrombosis [5].

Arterial thrombosis - The clinical problem

A balanced hemostatic system aims at preserving vascular integrity while maintaining blood fluidity. However, our modern lifestyle has caused a shift of the hemostatic equilibrium towards excessive thrombus formation. Today, ischemic vascular disease, including myocardial infarction, is the major cause of morbidity and mortality in the Western world [6], and is becoming a major health concern also in developing countries due to the adoption of a westernized lifestyle. These conditions are most often caused by excessive arterial thrombosis on the surface of an eroded or ruptured atherosclerotic plaque (reviewed in [7,8]). Loss of integrity of the inner layer of the vessel, the endothelium, causes platelets and the coagulation system to come in contact with thrombogenic sub-endothelial material, thereby initiating a thrombotic process. If the forming thrombus is not properly controlled and restricted in

size, the clot will eventually occlude the vessel, blocking normal circulation to down-stream tissue. This results in inadequate oxygen supply, tissue hypoxia and threatening tissue infarction. If this occurs in a coronary artery, death of the myocardium could lead to permanent damage of the heart with subsequent arrhythmias, heart failure, and severe reduction of heart function or even death. Given these widespread and severe consequences, studies of the underlying mechanisms leading to arterial thrombosis are warranted, as is investigating means to reduce the incidence of these events.

Arterial thrombosis - Clot formation

When a vessel is damaged, e.g. by plaque rupture, sub-endothelial structures, including collagen and tissue factor, are exposed to the components of the blood (reviewed in [8]). Platelets exposed to collagen and von Willebrand factor rapidly become activated and release factors including ADP and thromboxane A2. This leads to further activation, adherence, and aggregation of more platelets to the site of injury in a positive feedback loop. In parallel, exposure of sub-endothelial tissue factor in combination with circulating coagulation factor VII activates the extrinsic coagulation cascade and generates active factor X. This active form of factor X in combination with factor V then cleaves pro-thrombin to thrombin which leads to the formation of fibrin strands that stabilize the loose platelet plug. To further stabilize the clot, the fibrin threads are covalently cross-linked by the means of factor XIII. The activation and actions of the coagulation system are partly limited by factors of the anticoagulant pathways [9], including antithrombin, the protein C/protein S/thrombomodulin pathway, and the tissue-factor pathway inhibitor.

Arterial thrombosis - Clot dissolution

The fibrinolytic system

In vascular damage, once a blood clot has served its purpose to prevent blood loss and the vessel has healed, the clot has to be removed. This is accomplished by means of breakdown of the stabilizing fibrin network of the clot. The endogenous fibrinolytic system is responsible for fibrin dissolution (reviewed in [10,11]). This system is regulated both by factors circulating in plasma and factors released from the endothelium. The key event in initiating a fibrinolytic process in the vascular compartment is the local, regulated release of tissue-type plasminogen activator (t-PA) from endothelial cells (Figure 1) [12]. When t-PA is released it catalyzes the conversion of the inactive circulating zymogen plasminogen to plasmin, which in turn works as an enzyme that cleaves the stabilizing fibrin network of the clot causing the clot to disintegrate. The fibrinolytic system is regulated at several levels to ensure a localized fibrinolytic process. After its release, the majority of t-PA is rapidly inactivated by complex-binding to its main inhibitor, plasminogen activator inhibitor-1 (PAI-1), meaning that only a fraction of total circulating t-PA (approximately 20% [13]) is active. Both free and complex-bound t-PA is rapidly cleared by the liver. The activity of t-PA is also indirectly reduced by the action of thrombin-activatable fibrinolysis inhibitor (TAFI) which modifies the fibrin molecule making it more resistant to fibrinolysis [14]. The fibrin-degrading action of plasmin is mainly counteracted by the inhibitor α2-antiplasmin.

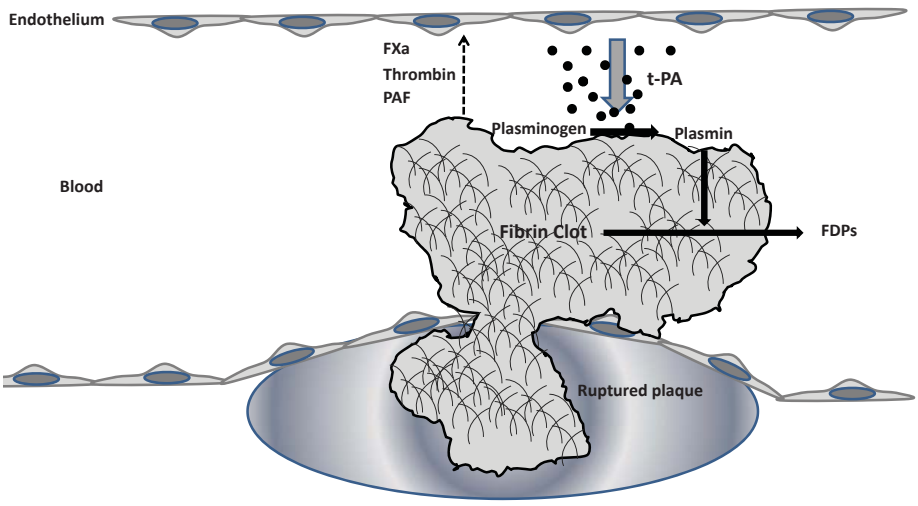


Figure 1. Schematic presentation of the intravascular fibrinolytic system. When a clot is forming on a ruptured plaque, factors from the clotting process including Factor (F) Xa, thrombin, and platelet-activating factor stimulate the endothelial cells to rapidly secrete t-PA from storage vesicles. t-PA then cleaves the pro-enzyme plasminogen to plasmin which in turn degrades the fibrin mesh of the forming clot. This system thus works as an important counter-regulatory mechanism to the clotting process to prevent occluding thrombosis. PAF: Platelet activating factor, FDP: Fibrin degradation products.

The thromboprotective response

The body's capacity to dissolve blood clots by fibrin breakdown is not only important to remove established clots formed after vascular damage, but the fibrinolytic system is also activated already at initiation of a clotting process to prevent excessive growth of the fibrin mesh into the intraluminal space. We and others hypothesize that this acute stimulation of fibrinolysis in a thrombotic situation, counterbalancing fibrin formation, constitutes a form of thromboprotective response of the vasculature. As such, this system has been postulated to be a central mechanism for prevention of thrombotic events. The thromboprotective role of the fibrinolytic system is supported by the observation that thrombi (e.g. on eroded plaques) can exist in an equilibrium, with a balance between fibrin formation and breakdown [7,15]. If not kept under control by the fibrinolytic thromboprotective response, such thrombotic situations would eventually propagate to occluding thrombosis. The significance of an active thromboprotective response is further corroborated by the observation that in approximately 30% of clinical occluding thrombotic events (myocardial or cerebral infarctions), the occluded artery spontaneously re-perfuses within the first hours [16,17].

The central role of t-PA in the thromboprotective response

The key player of the vascular fibrinolytic response is t-PA released from the endothelial cells (Figure 1). When t-PA is synthesized in the endothelium, a small portion is directly secreted *via* a constitutive pathway into the vascular space, maintaining an anti-thrombotic surface at the healthy vascular wall. However, the majority of newly synthesized t-PA is

not immediately secreted, but instead stored in specific storage granules close to the plasma membrane on the luminal side of the cell, enabling a rapid release of large amounts of clot-dissolving t-PA if a clotting situation should emerge (reviewed in [18,19]). This storage pool has been localized to Weibel-Palade bodies [20] as well as to other storage vesicles specific for t-PA [21], or recently, co-localized with various cytokines [22]. Stimuli for acute t-PA release include several products generated during the process of thrombus formation, including thrombin, bradykinin, factor X and platelet activating factor [23,24,25], as well as factors produced by tissue ischemia [26,27,28,29]. The process of acute release occurs within minutes of a clotting activation and can achieve high local t-PA concentrations, estimated to be similar or even higher than those achieved systemically in exogenous thrombolytic therapy [24,25]. However, in contrast to exogenous *post-hoc* administration of recombinant t-PA, endogenous acutely released t-PA is present during the active clotting process and, as such, is much more effective in mediating fibrin breakdown and clot lysis [30]. Taken together, this implies that the body has its own built-in clot-dissolving system able to prevent formation of occluding thrombi [31]. The timely response of the endothelium to the factors from a clotting process, and the amounts of t-PA available in the storage pools may be critical factors for the efficiency of this system, and thus for the outcome of a thrombotic event.

t-PA and risk for thrombotic events

Somewhat counter-intuitively, high circulating plasma levels of t-PA have consistently been associated with an increased risk for ischemic vascular disease, both in apparently healthy individuals and individuals with pre-existing arterial disease [32,33,34] (extensively reviewed in [35]). This apparent paradox could however be explained by the fact that systemic plasma t-PA levels, attained from a venous blood sample, reflect both the active (free) form of t-PA as well as the inactive form bound to PAI-1. Active t-PA is cleared from the circulation more rapidly than the t-PA/PAI-1 complex [36], and thus plasma t-PA levels mainly reflect the levels of plasma PAI-1. As a result, high plasma levels of t-PA are more indicative of hypofibrinolysis caused by high levels of circulating PAI-1, and do not predict the capacity for endothelial t-PA release [13]. More in line with its postulated role in the thromboprotective response, several studies that instead have assessed active t-PA have found low t-PA activity in plasma to correlate with an increased risk for ischemic vascular disease [37,38,39].

An even more accurate way to study fibrinolytic capacity is to measure the ability for local stimulated acute t-PA release, either by venous occlusion or by pharmacological activation of t-PA secretion in a particular vascular bed [13,40]. Using this local model, the clinical importance of a functional acute t-PA release in man has been confirmed by the observation that fibrinolytic capacity, measured by local t-PA release, is a predictor of future cardiovascular events in patients with coronary heart disease [41]. Moreover, the local release capacity of t-PA in humans has been found to be impaired by various factors associated with increased risk of thrombosis. These have been found to be of both hereditary, genetic nature as well as due to acquired, patho-physiological causes.

Genetic impairment

In support of the importance of a functional acute t-PA release capacity, several families with impaired capacity for release of t-PA from the vessel wall have been described to suf-

fer from early-onset, often recurrent, thrombotic events, mainly in the venous circulation [42,43,44,45]. However, the underlying specific genetic cause of the reduced t-PA release capacity in these families was not investigated in these early studies. In 1999 our group described that the t-PA *Alu* insertion/deletion polymorphism was associated with variable local t-PA release capacities [46], and in the following years that a polymorphism in linkage disequlibrium with the *Alu* I/D polymorphism (the -7,351 C/T polymorphism), located in an upstream GC-box, was associated with reduced transcription factor binding, t-PA expression, and release capacity [47,48,49]. This naturally occurring genetic "knock-down" of t-PA was later confirmed to be associated with a 3-fold adjusted increased risk for myocardial infarction [50], demonstrating the importance of a sufficient t-PA production in maintaining vascular patency also in the arterial circulation.

Functional impairment

Besides being genetically impaired in some individuals, t-PA release can also be functionally impaired. Interestingly, several risk factors associated with atherothrombotic disease have been shown to reduce the capacity for regulated t-PA release. Such a condition is hypertension [51,52,53], which is associated with a reduced capacity for t-PA release probably at least in part due to the increased mechanical stress on the endothelium [54,55]. Overweight/obesity, in particularly in combination with a sedentary life style, is also associated with reduced t-PA release [56,57,58]. Cigarette smoking has been shown to reduce t-PA release capacity both in the forearm [59,60,61] and coronary circulation [62]. Atherosclerotic coronary artery disease, as well as coronary atherosclerotic burden, has also been associated with reduced coronary t-PA release [62,63].

Impairment of t-PA in inflammation?

Of interest, all above-mentioned conditions have been associated with varying degrees of inflammatory stress [64,65,66,67,68]. Inflammation is widely considered to be an integral contributor to atherothrombotic disease. Inflammatory processes are involved in stimulating initiation, propagation and vulnerability of the atherosclerotic plaque (reviewed in [67,68,69]) as well as in promoting thrombosis by increasing levels of local and circulating coagulation factors (including tissue factor and fibrinogen) and reducing the effect of the anti-coagulant pathways thus inducing a hypercoagulable state (reviewed in [70,71,72,73]). In line with this inflammation, as measured by increased levels of circulating C-reactive protein (CRP), is predictive of myocardial infarction and stroke both in apparently healthy individuals and in individuals with established cardiovascular disease [74,75,76,77,78,79]. Also, patients with chronic inflammatory autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus have an increased risk of developing acute atherothrombotic disorders [80,81,82,83,84,85,86,87]. The recent observation that inhibition of TNF-α reduces the incidence of cardiovascular events in RA patients [88,89], suggests that the effect is at least partly mediated by pro-inflammatory cytokines.

However, inflammation might not only promote thrombosis by affecting a number of coagulation processes, it could also affect the fibrinolytic system [90]. Pro-inflammatory cytokines have been consistently shown to increase endothelial PAI-1 production *in vitro* [91,92,93,94,95,96,97]. Studies of the effects of inflammatory cytokines on t-PA production in cultured endothelial cells have, however, been somewhat conflicting, showing both re-

duced [92,94,97,98], increased [99] and unchanged [91,93,96] levels of t-PA antigen/mRNA after cytokine stimulation. This means that no consensus of the effect of pro-inflammatory cytokine exposure on endothelial t-PA production is available.

Regulation of endothelial t-PA synthesis

Data both from *in vitro* systems and experimental animal models indicate that the amount of t-PA that can be acutely released is largely dependent upon its rate of *de novo* synthesis [24]. If t-PA expression is increased in these models by pharmacological means, so is the amount stored in the releasable pools and as a consequence also the amounts released after stimulation. Conversely, if t-PA synthesis is reduced in these models, so is the release capacity. The validity of these experimental observations is supported by the fact that individuals with the low synthesis (T) genotype of the t-PA enhancer polymorphism also have a reduced t-PA release [47]. t-PA synthesis is considered to be regulated mainly on the transcriptional level [100], even though post-transcriptional regulation may occur in some settings. The classical transcriptional regulation of the t-PA gene has been extensively studied [100]. There are, however, also data suggesting that besides classical *cis*/*trans* regulation of the t-PA gene, an additional level of regulation, epigenetic regulation, could also be of importance.

Transcriptional regulation of t-PA - Classic regulation paradigm

Traditionally, in classical gene regulation, transcriptional activity is considered to depend mainly upon the binding of stimulatory or inhibitory transcription factors to certain regulatory DNA motifs in the gene regulatory regions [101], the so-called *cis*/*trans* model (Figure 2). The t-PA gene regulatory region contains two separate transcription initiation sites (TIS), located 110 bp apart. The upstream TATA-dependent site was initially believed to be the major initiation site [102,103], but later studies have shown that the down-stream TATA-less site appears to be the predominant one with an approximately ten-fold higher transcription rate in endothelial cells as compared to its TATA-dependent counterpart [104,105]. Positions in the t-PA gene in this thesis are therefore numbered relative to the TATA less site.

Several transcription factor binding *cis*-elements have been identified both within the proximal t-PA promoter and at locations further upstream (Figure 2A). TATA-less transcription is often driven by factors, including Sp-family transcription factors, assembling at GC-boxes [106]. In line with this, several GC boxes 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 [104,107,108], and a correlation between the binding of nuclear proteins to GC box III and t-PA expression has been reported for several cell types [109]. A CRE-like site (bp -223 to -216) has also been shown to be important for both constitutive and inducible activation of the t-PA promoter in endothelial cells [104]. This DNA element binds transcription factors belonging to the AP-1 and CREB/ATF families, but cell type specific binding has been described [104,107,110]. Moreover, a consensus site for the binding of transcription factor NF1 has been identified (bp -202 to -188) [107].

Besides the elements of the proximal t-PA promoter, *cis*-acting elements located further upstream have also been described. These include a functional κB element, which was recently identified in the t-PA gene of human neuronal cells (bp -3081 to -3072) [111]. The t-PA gene is also under control of a distant enhancer region located 7.1 to 8.0 kb upstream of the tran-

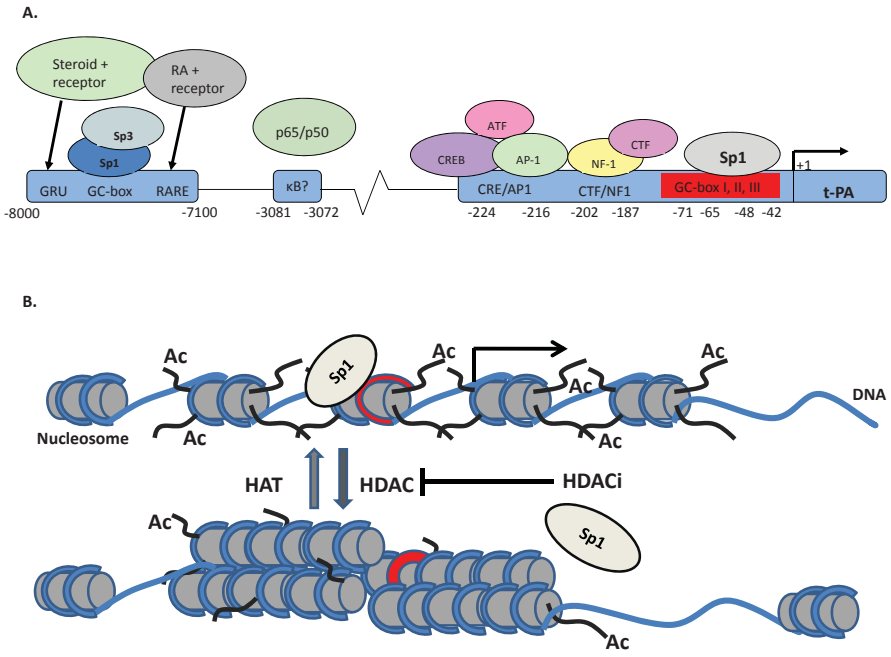


Figure 2. Regulation of the t-PA gene. **A**. t-PA gene regulation as depicted in classical gene regulation. In classical gene regulation, binding of transcription factors to elements in the gene regulatory region is considered. The DNA is depicted as a linear structure where the cis-elements are available for binding of various transcription factors. The two major regulatory regions of the t-PA gene, the proximal promoter region and the upstream enhancer region, are shown with transcription factors that have the potential to bind these elements. For example, Sp1 binds to the proximal GC-boxes (marked in red). **B**. Hypothetical epigenetic regulation of the t-PA gene. In epigenetic regulation the structure of the chromatin is also considered. Even though a cis-element is present in the DNA sequence, it may be more or less available for transcription factor binding depending on the local chromatin structure. This is partly regulated by the acetylation (Ac) state of histone proteins. Histone acetylation is regulated by the HATs and the HDACs. HDACis prevents histone deacetylation and thus causes the histones to be more extensively acetylated and the chromatin to become more accessible. The Sp1 factor and the GC-boxes (marked in red) are included as a theoretical example.

scription start site [112]. This region contains among other things an Sp1-binding GC-box involved in constitutive and induced t-PA production [108]. This GC-motif is the place for the -7,351 C/T polymorphism, where the wild-type cytosine nucleotide is exchanged for a thymidine, disrupting transcription factor binding and causing a reduction in t-PA production [48,49].

Transcriptional regulation of t-PA - Epigenetic regulation paradigm

In addition to the classical *cis*/*trans* paradigm for gene regulation, another "higher order" level of transcriptional control also exists. This so-called epigenetic regulation is not dependent on the actual DNA sequence but is considered to be a meiotically or mitotically stable level of regulation "over or above" (*epi*) the DNA genetics. This form of gene regulation is modifiable and epigenetic mechanisms may constitute the molecular pathways by which the environment can influence long term gene expression. Hence, epigenetic mechanisms have

been implicated in life-style associated modulation of cardiovascular disease susceptibility [113]. Given that t-PA production appears to be affected by a number of life-style factors, it is likely that an epigenetic component of t-PA gene regulation exists.

The DNA in each cell is, in linear condition, approximately 2 m long. In order to organize this long stretch of nucleotides into a nucleus that is a few μM in diameter, and still be able to fine tune the replication and transcription of all genetic material, the DNA is organized into a DNA/protein polymer called chromatin. The structure and packaging of chromatin is heterogeneous. It can exist in a relatively compacted and dense form (heterochromatin) which is associated with silent parts of the genome, and a more accessible form (euchromatin) associated with actively transcribed parts of the genome [114] (Figure 2B). There are several major epigenetic regulatory mechanisms affecting chromatin compaction and gene expression (reviewed in [115]), the two most extensively studied being DNA methylation and histone modifications.

DNA methylation

Methylation of DNA is considered a stable epigenetic modification responsible for keeping certain parts of the genome transcriptionally inactive/silent. DNA methylation primarily occurs on cytosine nucleotides that are positioned upstream of a guanine nucleotide (a "CpG site") yielding 5-methyl-cytosine [115]. There are several mechanisms by which methylation can lead to repression of gene expression [116], including sterical hindrance of transcription factor binding as well as heterochromatin formation. The group of Kruithof recently investigated the methylation status of the t-PA proximal regulatory region in endothelial cells and hepatocytes. They found that in endothelial cells, the CpG motifs closest to the t-PA TIS were to a high degree unmethylated. This was in contrast to primary hepatocytes which had a higher degree of methylation in the proximal CpG motifs, and a hepatocyte cell line which was even more extensively methylated in this region. Interestingly, the degree of methylation of the proximal CpG sites in these cell types corresponded to levels of t-PA expression [117].

Histone modifications

During the last decade the important role of the DNA scaffold histone proteins in gene regulation has been increasingly recognized [114]. The basic unit of chromatin is the nucleosome, a complex of histone protein subunits around which approximately 147 bp of DNA is wrapped almost two turns [114]. The nucleosome core particle consists of two copies each of the core histones H2A, H2B, H3 and H4. The nucleosome is a condensed structure, but the N-terminal parts of the histone proteins protrude from the tight core nucleosome structure as "tails" and are readily available for post-translational modifications. Histone H3 and H4 tails, in particular, are subject to an array of post-translational modifications (including phosphorylation, acetylation, mehtylation and ubiqutination) that, collectively, affects the relative compaction of the chromatin and hence the accessibility of transcription factor binding sites [114,118]. There are two main theories regarding the mechanism of histone modification-mediated gene regulation [114]. The original theory suggested that post-translational modifications may affect electrostatic interactions between the histone tails and DNA to "loosen" chromatin structure. However, more recently it was proposed that specific combinations of these modifications may create binding epitopes where modification-specific regulatory proteins, including chromatin remodeling enzymes, may bind and structurally perturb the chromatin, the so-called "histone code" hypothesis [119,120] .

One of the most widely studied histone modifications is acetylation of histone lysine residues. Histone acetylation status is regulated by the concerted action of two enzyme families, the histone acetyl transferases (HATs) which catalyze the addition of acetyl groups, and the histone deacetylases (HDACs) which remove subsequent acetyl groups [121] (Figure 2B). Chromatin containing a high degree of acetylated histones is generally considered to be open or permissive, whereas a low acetylation level is indicative of a closed or repressive chromatin [122].

There appears to be certain genes that are sensitive to regulation by changes in histone acetylation whereas others are not. Transcription profiling experiments have shown that somewhere around 2-5% of all genes are affected by agents modulating histone acetylation [123]. Two early studies by the group of Kooistra implied that the t-PA gene might belong to the group of genes sensitive to regulation by histone acetylation [124,125]. Endothelial cells were treated with butyrate and trichostatin A (TSA), two substances known to inhibit HDAC enzymes and cause relative hyperacetylation of histones. Both these substances increased t-PA mRNA and protein production. These findings were recently confirmed and extended by the group of Kruithof [117]. Interestingly, it is possible that this potential epigenetic regulation of the t-PA gene could be utilized to stimulate t-PA synthesis, and thereby release capacity, in man when suppressed by patho-physiological factors. This could perhaps be used as a novel preventive strategy for arterial thrombosis.

Stimulation of endogenous t-PA production - A novel prevention for arterial thrombosis?

Current clinical approaches for prevention of atherothrombosis are mainly focused on preventing the initiation of thrombus formation by reducing excessive platelet aggregation, and in some situations also by inhibiting the coagulation system [126] (Figure 3). However, despite these preventive strategies arterial thrombotic events are common [127], indicating that targeting platelets and/or the coagulation system may not be sufficient and that, in addition, it could be beneficial to target mechanisms for dissolution of the clot as well.

A highly interesting possibility to strengthen the fibrinolytic part of the hemostatic equilibrium would be to pharmacologically enhance the endogenous capacity for endothelial t-PA release, in particular in high-risk patients with an impaired t-PA response. Such a preventive strategy would thus restore or potentiate the ability of the vasculature to rapidly and efficiently "self-medicate" in an acute thrombotic situation. As the release capacity appears to be tightly linked to the amount available in the storage pools, and thereby to the synthesis rate of t-PA [24], a means to stimulate t-PA synthesis could shift the hemostatic balance in favor of mechanisms maintaining vascular patency. In comparison to pharmacological treatment with recombinant t-PA, stimulating endogenous t-PA production would likely have two major advantages. First, recombinant t-PA treatment is by necessity initiated with a significant delay with respect to the clotting event, i.e. when the clot has already occluded the vessel giving rise to clinical symptoms. This late onset of recombinant t-PA treatment impairs its efficacy as t-PA needs to be present during, rather than after, clot formation in order to initiate an effective fibrinolytic response [30,31]. Second, since recombinant t-PA is administrated intravenously, t-PA levels are increased throughout the circulation which

causes a considerable risk of severe bleeding complications from other organs. In fact, the increased risk of bleeding prevents the use of thrombolytic therapy in several patient groups [128]. Both these major drawbacks of thrombolytic therapy could likely be avoided if instead the efficacy of the endogenous fibrinolytic system could be enhanced.

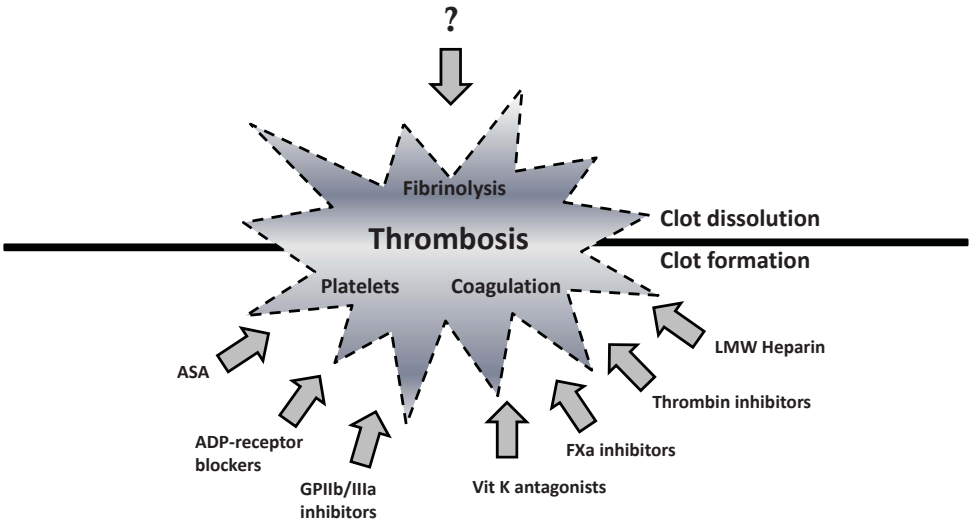


Figure 3. Current cardiovascular prevention regimes. The hemostatic system has three major components: the platelets, the coagulation system, and the fibrinolytic system. The cardiovascular prevention/treatment used today aims at inhibiting platelet aggregation most commonly by use of acetylsalicylic acid (ASA), ADP-receptor blockers and GPIIb/IIIa receptor antagonists, as well as reducing the activity of the coagulation system by e.g. vitamin K antagonists, low molecular weight (LMW) heparin, thrombin inhibitors and FXa inhibitors. No pharmacological treatment available today targets the fibrinolytic system.

Various ways to increase human endothelial t-PA synthesis have been explored in cultured endothelial cells (reviewed in [19]), and a number of substances inducing t-PA synthesis identified, including activators of protein kinase C (e.g. phorbol esters), cholera toxin, and retinoids. Some of these have also been shown to increase t-PA production in experimental animal models [18]. However, none of these substances are suitable for use in humans due to toxicity or stability problems. Statins, well-tolerated agents widely used for cholesterol lowering, have been shown to increase t-PA expression *in vitro* [129,130], but in higher concentrations than those achieved in plasma during cholesterol-lowering therapy in man. In line with this, no effect of Pravastatin-use on t-PA release capacity could be detected in a local release model [131]. Butyric acid has been shown to be one of the most potent stimulators of t-PA expression available, possibly achieving this effect *via* its HDAC-inhibitory activity [125]. However, this substance is not suitable for use *in vivo* due to its poor pharmacokinetic properties. During the last decade, a large number of new HDAC inhibitors (HDACis) have been developed for use in man, mainly for cancer treatment, but their effect on t-PA synthesis is unknown. If they indeed were to increase t-PA expression it is possible that these substances could be interesting candidates for stimulation of endothelial t-PA synthesis in man.

AIM

In the light of the background described herein, two aims for this thesis were formulated:

- To further evaluate the role of the pro-inflammatory cytokines Tumor Necrosis Factor-α (TNF-α), Interleukin 1-β (IL-1β) and Interleukin-6 (IL-6) on t-PA expression in cultured endothelial cells and investigate intracellular signaling mechanisms of importance (Study I and II)

- To evaluate different HDAC inhibitors as potential stimulators of t-PA production in endothelial cells, and further investigate the mechanisms of HDACi-dependent t-PA stimulation (Study III and IV)

MATERIALS AND METHODS

Cell culture and experimental design

Cell culture

The experiments presented in Studies I-IV were carried out on cultured human umbilical vein endothelial cells (HUVEC), and certain experiments were verified in human aortic endothelial cells (HAEC) or human coronary artery endothelial cells (HCAEC). HAECs and HCAECs were purchased from Lonza, while HUVECs were isolated from fresh umbilical cords obtained from normal deliveries at the maternity ward of the Sahlgrenska University hospital. HUVECs were prepared by mild 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. HUVECs and HAECs were maintained in EGM-2 complete culture medium, consisting of EBM-2 basal medium (Lonza) supplemented with 2% fetal bovine serum and growth factors (SingleQuots kit; Lonza) in plastic culture flasks at 37°C in a humidified 5% CO_2 incubator. For culture of HCAECs, EGM-2 medium was supplemented with additional fetal bovine serum to a total serum content of 5%. The medium was replaced every 2-3 days and sub-cultures were obtained by trypsin/EDTA treatment of confluent monolayers. HUVECs and HAECs/HCAECs were used in experiments at passage 1-2 and 4-6, respectively.

Experimental design

Study I and II

These studies aimed at determining the effect of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 on the expression of t-PA in endothelial cells and to study the underlying mechanisms of cytokine-regulated t-PA gene expression. HUVECs were seeded in plastic culture plates or plastic culture flasks and grown to confluence. Confluent HUVECs were exposed to 0.1-10 ng/ml of human recombinant TNF-α (Sigma-Aldrich), 1-100 pg/ml of recombinant human IL-1β, or 1-100 ng/ml of recombinant human IL-6 (both from R&D systems) in complete medium. After incubation with the cytokines for up to 48 h, cells and conditioned media were harvested. Cells that were used for collection of conditioned media were re-stimulated after 24 h in order to collect media from the time period 24-48 h. For the inhibition experiments, fresh complete EGM-2 medium with or without signaling pathway inhibitors were added to the cells 1 h before stimulation with 1 ng/ml of TNF-α or 10 pg/ml of IL-1β for 24 h. As the optimal inhibitor concentration can vary in different cell culture systems, the optimal concentration for each inhibitor was carefully titrated. Parthenolide (6-10 μM depending on batch) (Sigma-Aldrich) was used to inhibit NF-κB signaling, SB203580 (25 μM) (Biosource) to inhibit p38 MAPK, SP600125 (10 μM) (Calbiochem) to inhibit JNK and PD98059 (10 μM) (Biosource) to inhibit extracellular signal-regulated kinase (ERK)1/2 signaling. For the soluble IL-6 receptor (sIL-6R) experiments, HUVECs were incubated with 500 ng/ml sIL-6R (R&D Systems) for 24 h in the presence or absence of 10 ng/ml of recombinant human IL-6, or in combination with 10-50 μg/ml anti-IL-6 antibody (Pierce Bio-

technology). A summary of the methods used is presented in Figure 4. 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 was relevant also on the level of t-PA secretion. Western blot with phospho-specific antibodies was used to study activation of intracellular signaling pathways. Flow cytometry analysis was used to determine surface expression of IL-6Rα and gp130. Cell cultures were performed in duplicate and all experiments were performed on HUVECs from a minimum of 3 individuals unless otherwise stated in the figure legends.

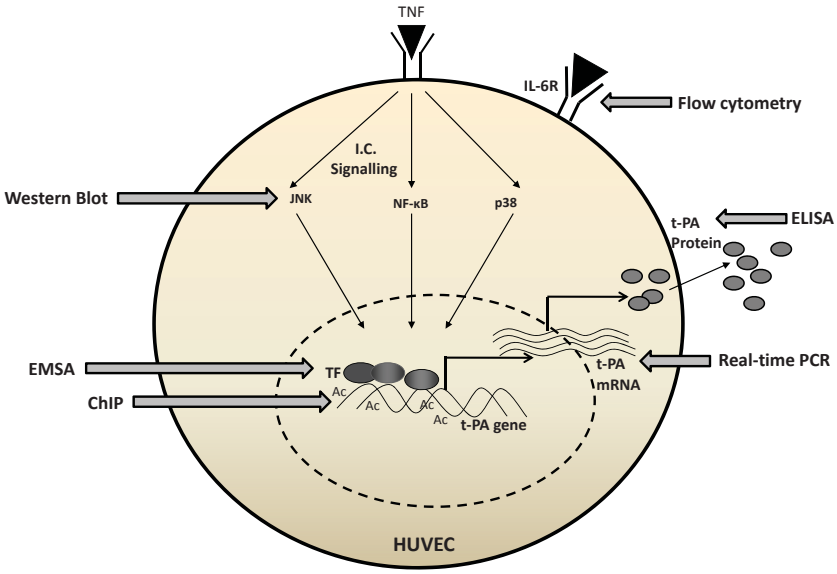


Figure 4. Overview of the methods used in this thesis. Western blot was used to study protein phosphorylation and activation of the different signaling pathways after cytokine stimulation (Study I and II). EMSA was used to study transcription factor interaction with elements of the t-PA promoter (Study I and II). ChIP was used to study the acetylation state of histones associated with the t-PA promoter (Study IV). Flow cytometry (FACS) was used to study the presence of the IL-6 receptor on HUVEC cells (Study II). Real-time PCR was used to quantify levels of t-PA mRNA (Study I-IV) and ELISA to quantify secreted t-PA protein (Study I-IV).

Study III and IV

These studies aimed at determining the effect of HDACis on the expression of t-PA in endothelial cells and to investigate if the profound effect of valproic acid (VPA) on t-PA production in endothelial cells was specifically related to its HDAC-inhibitory function. HUVECs were seeded in plastic culture plates or plastic culture flasks and grown to confluence. Confluent HUVECs were exposed to optimal concentrations of the following HDACis in complete medium: Na-valproate (Sigma-Aldrich), Phenylbutyrate (Enzo Life Sciences), Vorinostat (SAHA), Belinostat (PXD101), Givinostat (ITF2357), Panobinostat (LBH589), JNJ26481585, SB939, Mocetinostat (MGCD0103), and Entinostat (MS-275) (all from Selleck Chemicals), and Apicidin (Sigma-Aldrich). All the HDACis were protected from light and diluted in complete endothelial cell culture medium immediately before use. Cells were also exposed to the SIRT-inhibitors Splitomicin (Sigma-Aldrich) and EX-527 (Cayman

Chemical). After incubation with the substance for up to 72 h (with fresh medium and inhibitors added every 24 h), cells and conditioned media were harvested. For the TNF-α experiments, confluent cells were pre-incubated with 0.1 ng/ml of TNF-α for 24 h after which HDACis and fresh TNF-α were added for an additional 24 h. Cell cultures were performed in duplicate and all experiments were performed on cells from a minimum of 3 individuals unless otherwise stated. Regulation of t-PA mRNA expression was analyzed by real-time RT-PCR and t-PA protein secretion in conditioned media by ELISA. The effect of VPA on global histone acetylation was determined by western blot and on local histone acetylation surrounding the t-PA transcription initiation site by chromatin immunoprecipitation (ChIP) (Figure 4). The influence of specific HDAC enzyme knock-down on constitutive as well as VPA stimulated t-PA expression was investigated using siRNA transfection. The effect of VPA on global gene expression in HUVEC was determined by microarray analysis.

Analyzing techniques - Principles and methods

Real-time PCR

Principle

Real-time RT-PCR was used to quantify the levels of specific mRNA transcripts as a measure of transcription and gene activity (Study I-IV). The principle of this method is that the mRNA transcript pool in a cell is purified and reverse transcribed to cDNA. The specific transcript of interest is then amplified in a PCR reaction containing a dual-labeled probe. When this fluorescently labeled probe is hybridized to its target sequence during PCR, the Taq polymerase cleaves the reporter dye from the probe releasing the reporter dye into solution where the increase in dye emission is monitored in real-time and the threshold cycle analyzed. 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 [133]. 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 using the comparative C_T method (User Bulletin #2, Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous reference gene for Study I and II, and Hypoxanthine phosphoribosyl transferase (HPRT) for Study III and IV. GAPDH and HPRT are constitutively expressed genes, not affected by the specified treatments, and thus work as internal standards to correct for potential variation in RNA loading, cDNA synthesis, or efficiency of PCR amplification.

Method

Following experiments in which endothelial cells had been stimulated with pro-inflammatory cytokines (Study I-II) or HDACis (Study III-IV), total RNA was isolated from the cells using RNeasy Mini Kit (Qiagen) and potential genomic DNA contaminations were removed by treatment with DNase (Qiagen). mRNA was converted to cDNA with GeneAmp RNA PCR kit (Study I-II) or High Capacity RNA to cDNA Mastermix (Study III-IV) (both from Applied Biosystems). Levels of t-PA, PAI-1, VCAM-1, ICAM-1, u-PA, and vWF mRNA were analyzed with real-time RT-PCR, performed on a ABI Prism 7700 Sequence Detection System (Applied Biosystems) (Study I-II) or an Applied Biosystems 7500 Fast Real-Time PCR System (III and IV) using cDNA and Taqman reagents obtained from Applied

Biosystems. Oligonucleotide primers and Taqman probes for quantification of t-PA, PAI-1, and GAPDH mRNA were designed from the GenBank database using Primer Express version 1.5 (Applied Biosystems), whereas VCAM-1 and ICAM-1, vWF, uPA, and HPRT mRNA were quantified with Taqman® pre-designed gene expression assays™ (Applied Biosystems). To avoid amplification of genomic DNA, primer pairs were selected in order for the amplicon to span an exon junction. All probes designed in-house were dual-labeled with 5′-reporter dye FAM (6-carboxy-fluorescein) and 3′-quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine).

Enzyme-linked Immunosorbent Assay (ELISA)

Principle

ELISA was used to quantify secreted t-PA antigen after various stimulations (Study I-IV). The principle of this assay is that the samples, or a standard containing human recombinant t-PA protein, are added to wells that are coated with anti-t-PA IgG antibodies. After t-PA in the sample or standard has been allowed to bind to the antibodies, peroxidase-labeled anti-t-PA IgG is added. Wells are washed to remove unbound antibodies and peroxidase substrate is added. The peroxidase enzyme then converts the substrate to a colored product (directly proportional to the amount of protein present in the sample) which is quantified by spectrophotometry.

Method

The concentration of t-PA antigen in conditioned cell culture medium was determined with a commercially available ELISA (TriniLize t-PA) from Trinity Biotech. All samples were assayed in duplicate according to manufacturer's protocol.

Flow cytometry

Principle

Flow cytometry was used to detect potential surface expression of the IL-6 receptor subunits CD126 (IL-6Rα) and CD130 (IL6-Rβ) on HUVECs (Study II). The principle of flow cytometry is that fluorescently labeled antibodies to specific cell surface markers are added to the cells and are used to identify cell populations or examine the presence of a specific marker on a specific cell type. The flow cytometer, in addition to detecting cell size and granularity, also detects the fluorescence emitted from the labeled antibodies and can thus determine which cells that are staining positive for a certain marker. In order to determine the cut-off for negative/positive staining, isotype control antibodies (i.e. antibodies of the same isotype with no relevant specificity) are used to identify the location of the negative population in the diagrams.

Method

HUVECs were incubated with mouse monoclonal antibodies to CD126 (PE-conjugated) or CD130 (biotinylated) (both from BD Biosciences) or matching isotype control antibodies for 20 min at 4°C. The staining was discontinued by washing and, to samples containing biotinylated antibodies, streptavidin-conjugated allophycocyanin (APC) was added and the cells

incubated for an additional 20 min. Cells were then washed and cell pellets re-suspended and fixed in PBS containing 1% fetal bovine serum, 0.5 mM EDTA, 0.1% NaN_3 and 2% formaldehyde. 10 000 to 50 000 cells were analysed with a FACS Calibur flow cytometer equipped with CellQuest software.

Western blot

Principle

Effects of TNF-α or IL-1β on the activation of the NF-κB, ERK1/2, p38 MAPK and JNK pathways (Study I and II) as well as the effect of VPA on histone acetylation (Study IV) were evaluated by western blot. The principle of western blot is that total cell proteins are separated according to size on a denaturating SDS-PAGE gel, transferred to a membrane by electroblotting, and identified using specific antibodies. A secondary antibody conjugated to a peroxidase enzyme is then added and upon addition of a substrate, a chemiluminescent signal is emitted which can be detected by an imaging system.

Method

Stimulated HUVECs were harvested in Laemmli sample buffer (Bio-Rad) with 5% β-merkaptoethanol, sonicated and boiled before being applied to a 10% (Study I and II) or 10-20% gradient (Study IV) pre-cast Tris-Glycine gel (Lonza) and electrophoresed in running buffer (Bio-Rad). Resolved proteins were transferred by blotting onto Hybond-P polyvinylidene fluoride membranes (Amersham Biosciences). Membranes were blocked and then incubated over night with primary antibodies of optimal dilution for each target. For Study I and II primary antibodies were directed against the phosphorylated or total forms of p65 (NF-κB subunit), JNK, p38 MAPK and ERK1/2. In Study IV, antibodies were directed against pan-acetylated H3 and pan-acetylated H4 as well as to total histone H3 and H4. After extensive washing, membranes were incubated with secondary antibody (anti-rabbit IgG, horseradish peroxidase linked) for 1 h at room temperature. Proteins were visualized using SuperSignal Chemiluminescent Substrate (Pierce Biotechnology).

Electrophoretic Mobility Shift Assay (EMSA)

Principle

EMSA was used to detect interactions between nuclear proteins and gene regulatory elements in the t-PA promotor (Study I and II). The principle of EMSA is that a radioactively (^{32}P) labeled DNA fragment containing the element of interest is incubated with isolated nuclear proteins/protein complexes. The DNA/protein complexes are then separated from each other and from free probe on a non-denaturing polyacrylamide gel. This generates a band pattern that can be interpreted and compared between treatments. To analyze the specific components of the protein complexes bound to the probe, antibodies are added. Binding of these antibodies increases the size of the complex which then will migrate slower through the gel and hence give rise to a different pattern, a so-called supershift.

Method

Two double-stranded oligomers 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 [111], and the t-PA cyclic adenosine monophosphate (cAMP) response element (CRE)-like site [104]. Labeling of the oligomers was carried out as described using T4 polynucleotide kinase and [γ-^{32}P]ATP [134,135]. 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 purified by electrophoresis, visualized by autoradiography, excised and eluted overnight. Supernatant solutions containing the labeled oligomer were precipitated and re-suspended to approximately 1000 cps/µl.

The preparation of nuclear extracts from HUVECs was performed as previously described [135] and protein concentrations were quantified using Bio-Rad reagents on a microplate reader (FLUOstar Optima; BMG LabTechnologies). Binding reactions were carried out in a volume of 10 µl containing 5 µg crude nuclear extract and 100 cps ^{32}P-labeled probe as described in more detail in Study I. The binding reactions were analyzed by electrophoresis in a 5% native polyacrylamide gel, and visualized by autoradiography. 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 p50, p65 and c-Rel [136], and against HUVEC t-PACRE binding proteins cAMP-responsive-element-binding protein (CREB), activating transcription factor 2 (ATF-2) and c-jun [104].

Chromatin Immunoprecipitation (ChIP)

Principle

ChIP was used to investigate the acetylation status of histones associated with the t-PA promoter (Study IV). By using the ChIP method, it is possible to study DNA-protein interactions in the living cell, i.e. with an intact chromatin structure. In ChIP, any DNA-protein interactions in the cell are fixed using formaldehyde which is added directly to the living cells. The fixed chromatin is then sheared to optimal fragment length by sonication. Antibodies to a specific protein/modification are then added and the proteins and cross-linked DNA fragments are enriched using protein A-labeled magnetic beads. After extensive washing, bound DNA fragments are eluted by protease degradation of the protein/antibody. Enriched DNA is then purified and the DNA of interest amplified by real-time PCR. The real-time PCR signal is proportional to the number of proteins of interest that bound the specific region amplified. Quantification is achieved by comparing the precipitated material to two controls: one "input control" which is chromatin which has not been subjected to an immunoprecipitation (IP), and one "no-antibody control" or "isotype antibody control" which is chromatin which has been subjected to the IP process without antibody/with an isotype control antibody (thus yielding the non-specific background). The relative protein binding to a region is then expressed as percent of input DNA corrected for background binding. Treated and untreated cells are subjected to the same IP-process and their relative enrichment of the protein of interest is compared.

Method

Confluent HUVECs were stimulated with VPA or control medium for 24 h. After washing, formaldehyde fixing (1% formaldehyde in PBS, 5 min), glycine quenching, and further wash-

ing steps, cells were lysed and immediately sonicated to shear chromatin to a length of 100-500 bp (as determined by capillary electrophoresis on an Agilent 2100 bioanalyzer) using a Diagenode Biorupture. Insoluble material was removed by centrifugation and chromatin concentration was determined using the Quant-iT dsDNA BR kit and Qubit fluorometer (Invitrogen Life Technologies). Immunoprecipitation was performed on 1 µg of sheared chromatin, corresponding to approximately 5 x 10^5 cells per IP. Chromatin was diluted to 500 µl and pre-cleared by the addition of 20 µl Protein A-coupled magnetic beads (Invitrogen) for 2 h on constant rotation. Four µg of each antibody and 20 µl of protein A beads were pre-incubated for 2 h before the addition of the pre-cleared chromatin and the reactions were left over night at 4°C on a rotating platform. The antibodies used were pan acetylated histone H3 (K9, 14, 18, 23, and 27) (Active Motif) or pan acetylated histone H4 (K5, 8, 12, 16) (Millipore). The following mono-lysine acetylation modifications were also detected using specific antibodies for each modification: acH3K9, acH3K14, acH3K18, acH3K23, acH3K27 and acH4K5, acH4K8, acH4K12, and acH4K16. A no-antibody control reaction was included in each run. After extensive washing, captured DNA was eluted from the beads by proteinase K digestion and purified using spin column purification (Nucleospin Extract II, Macherey-Nagel). Isolated DNA fragments were quantified using real-time PCR with SYBR Green detection and the following primers spanning the t-PA major transcription initiation site (-46 to +92): Forward primer 5'-ACCCCCTGCCTGGAAACTTA-3' and reverse primer 5'-GGTACAGAAACCCGACCTACCA-3'.

Short interfering RNA transfections

Principle

In order to determine the potential relevance of different class I HDACs, each class I HDAC enzyme was independently depleted with short interfering RNA (siRNA). siRNA transfection is used to transiently and specifically silence the expression of a gene and thereby to shut down the production of a specific protein of interest. Short double-stranded oligonucleotides (21-23 nucleotides long) are introduced into the cell by means of transfection. These siRNA oligonucleotides then associate with the RNA induced silencing complex (RISC) and guides this complex to the complementary mRNA transcripts, where the complex cleaves and destroys the mRNA molecules resulting in a knock-down of the production of the specific protein.

Method

siRNA (ON-TARGETplus SMART pool siRNA sets) specific for class I HDACs (HDAC1, HDAC2, HDAC3 and HDAC8) were obtained from Dharmacon. HUVECs were plated the day before transfection in 24-well plates in EGM-2 medium without antibiotics and incubated overnight. The following day, siRNA (final concentration 10 nM) and DharmaFECT 4 transfection reagent (Dharmacon) in OptiMEM medium (Invitrogen) were combined and added to the cells. Cells were re-transfected according to the same protocol 24 h later. Forty-eight hours after the second siRNA transfection, the cells were treated with VPA or control medium and 24 h later mRNA was extracted and analyzed by real-time PCR to determine target mRNA reduction and t-PA mRNA expression. Results were only used when target reduction was over 80%. Two negative controls were used for siRNA, in one the Dharma-

FECT 4 transfection reagent was added alone to cells (mock), in another a control siRNA was used (All Star Negative control, Qiagen). No difference in expression of t-PA or target gene was observed with either control.

Microarray - Transcription profiling

Principle

The effect of VPA on global gene expression in HUVEC was determined by microarray analysis (Study IV). The principle of global expression analysis is that thousands of DNA probes are attached to a solid surface in an ordered fashion. In the case of the Affymetrix Human Gene 1.0 ST microarray used here, the array contains 764 885 distinct oligonucleotide probes attached to the chip surface, representing 28 869 genes. This chip represents a whole-transcript expression analysis meaning that the probes (in average 26 probes/gene) span the entire gene instead of only the 3' portion as in previous 3' based expression arrays. Purified RNA from samples and non-treated controls is converted into cDNA in a two-step model which amplifies the initial RNA sample. This cDNA is then fragmented, biotin labeled, and hybridized to the array, one sample per array. After washing, binding of biotinylated cDNA to the probes is detected by the addition of streptavidin coupled to a fluorescent dye. After streptavidin binding and washing, the intensity of the fluorescent signal for each probe is detected and is proportional to the relative expression level of the corresponding gene.

Method

Gene expression in VPA-treated and untreated HUVECs from 4 donors was analyzed using the Human Gene 1.0 ST microarray (Affymetrix). Target preparation and hybridization to the DNA microarray were performed according to standard Affymetrix protocols at the Uppsala Array Platform (Uppsala, Sweden). Raw data were analyzed using the RMA (robust multi-array average) method implemented in the Affymetrix software Expression Console. Probe sets with a log2 ratio above +1 or below -1 and a significantly changed expression ($p<0.05$, false discovery rate (FDR) adjusted p-value) were classified as regulated.

In wanting to focus on the effect of VPA on the hemostatic system, hemostasis genes were identified using the Amigo database (http://amigo.geneontology.org).

Statistics

Data throughout this thesis are presented as mean and standard error of the mean (SEM). The statistical evaluation was performed using a paired Student's t-test unless otherwise stated in the figure legends. All relevant comparisons are specified in the figure legends and were performed between samples from the same experiment and time-point to check for statistical significance. A p-value of less than 0.05 was considered significant. Changes across dose (Study III) or time (Study IV) were analyzed by ANOVA for repeated measures using the PASW v. 18 software.

RESULTS AND DISCUSSION

Study I and II - Role of inflammatory cytokines in regulation of endothelial t-PA production

Inflammation has an established role as a risk factor for cardiovascular disease, and pro-inflammatory cytokines have been described to affect several aspects of atheroma development and thrombus formation. However, cytokines may also affect the fibrinolytic system. The role of cytokines in the induction of PAI-1 appears clear; however the effect of inflammatory mediators on t-PA production is more uncertain. Thus, we investigated the effects of the inflammatory cytokines TNF-α, IL-1β and IL-6 on t-PA gene and protein expression in endothelial cells, and studied by which signaling mechanisms the effects are mediated.

Finding 1: Prolonged TNF-α and IL-1β exposure suppresses t-PA production in endothelial cells

Effects of the pro-inflammatory cytokines TNF-α and IL-1β on t-PA expression in HUVECs were investigated. Cells were exposed to varying concentrations of the cytokines for 6-48 h and t-PA mRNA and protein levels were quantified. At the earliest time point (6 h) only modest effects on t-PA mRNA were seen. TNF-α and the highest concentration of IL-1β did not affect mRNA levels, whereas a small but significant suppression of t-PA mRNA could be seen with the lower doses of IL-1β (Figure 5 A and C). However, after extended incubation with the cytokines (24-48 h) both TNF-α and IL-1β caused a dose-dependent pronounced suppression of t-PA mRNA levels to approximately 20-50% of those in control cells (Figure 5 A and C). This suppression was also evident on the protein level as t-PA antigen secreted to the culture medium was comparably reduced. However, the effect on protein secretion was delayed compared to the effect on mRNA, being most evident during the 24-48 h period when t-PA protein secretion was suppressed to about 30-60% of normal (Figure 5 B and D).

Previous studies of the effects of inflammatory cytokines on t-PA production in endothelial cells have shown somewhat conflicting results. Regarding TNF-α, there are three previous reports describing reduced-PA production [92,97,137] and one describing an induction [99]. For IL-1β previous studies mainly focused on the effect on PAI-1 and on t-PA antigen or activity [91,93,94,95,96,97,98,138,139]. These studies have shown a consistent increase of PAI-1 expression and/or activity in endothelial cells of different vascular origins. However, regarding t-PA the reported effects of IL-1 have been somewhat divergent. Most studies using IL-1β (or the IL-1 receptor agonist IL-1α) have reported unchanged levels of t-PA antigen/activity upon stimulation [91,95,96,98,139], whereas two studies showed a decrease of secreted and cell-associated t-PA antigen and activity after treating endothelial cells with IL-1 [94,97]. In a recent study, CRP was found to inhibit t-PA antigen and activity in HAECs *via* the generation of IL-1β and TNF-α, although the authors state that this is due to a post-transcriptional effect of the cytokines on t-PA as they did not detect a reduction in t-PA mRNA [140].

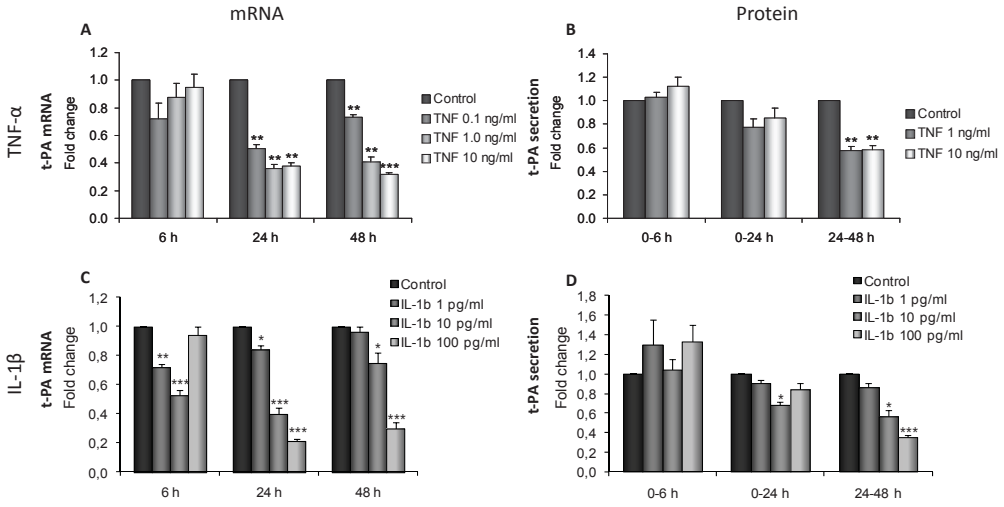


Figure 5. Prolonged TNF-α and IL-1β stimulation suppress t-PA mRNA expression and protein secretion in endothelial cells. HUVECs were exposed to 0.1-10 ng/ml of TNF-α or 1-100 pg/ml of IL-1β for 6-48 h and analyzed by real-time PCR and ELISA. **A**. Effect of TNF-α on t-PA mRNA expression. **B.** Effect of TNF-α on t-PA secretion. **C**. Effect of IL-1β on t-PA mRNA expression. **D**. Effect of IL-1β on t-PA protein secretion. *n*=3.
*p<0.05, **p<0.01, ***p<0,001

The discrepancy between our results and some of the previous studies [91,95,96,98,99,139] could reflect a difference in the duration of cytokine stimulation. One important finding in our studies is that the suppressive effect of pro-inflammatory cytokines on t-PA seems to be of slow kinetics. Whereas the majority of earlier studies investigated t-PA antigen levels at time points ≤24 h, we here show that the suppressive effect on t-PA, although detectable on the RNA level after 24 h, is evident only after 24-48h on the protein level. Indeed, when performing tests at earlier time points (1.5 and 3 h), we found that short term incubation of endothelial cells with high concentrations of IL-1β is associated with a transient increase of t-PA mRNA production (data not shown), consistent with previous findings [99]. This transient t-PA induction could perhaps also be an explanation for the somewhat varying dose-response patterns seen at 6 h on the RNA level and at 24 h at the protein level. If the high concentrations caused a transient stimulatory response this effect may linger, especially on the protein level where accumulated t-PA release for the first 24 h is measured.

Finding 2: IL-6 has the capacity to suppress t-PA production if the IL-6 receptor component sIL-6Rα is present

Besides TNF-α and IL-1β, IL-6 is a classical pro-inflammatory cytokine produced by macrophages, T cells and also by cells of the vascular wall including smooth muscle cells [69,141]. In order to determine the effect of this cytokine on t-PA expression, HUVECs were exposed to 1-100 ng/ml of IL-6 for 6-48 h. In contrast to TNF-α and IL-1β, we were unable to de-

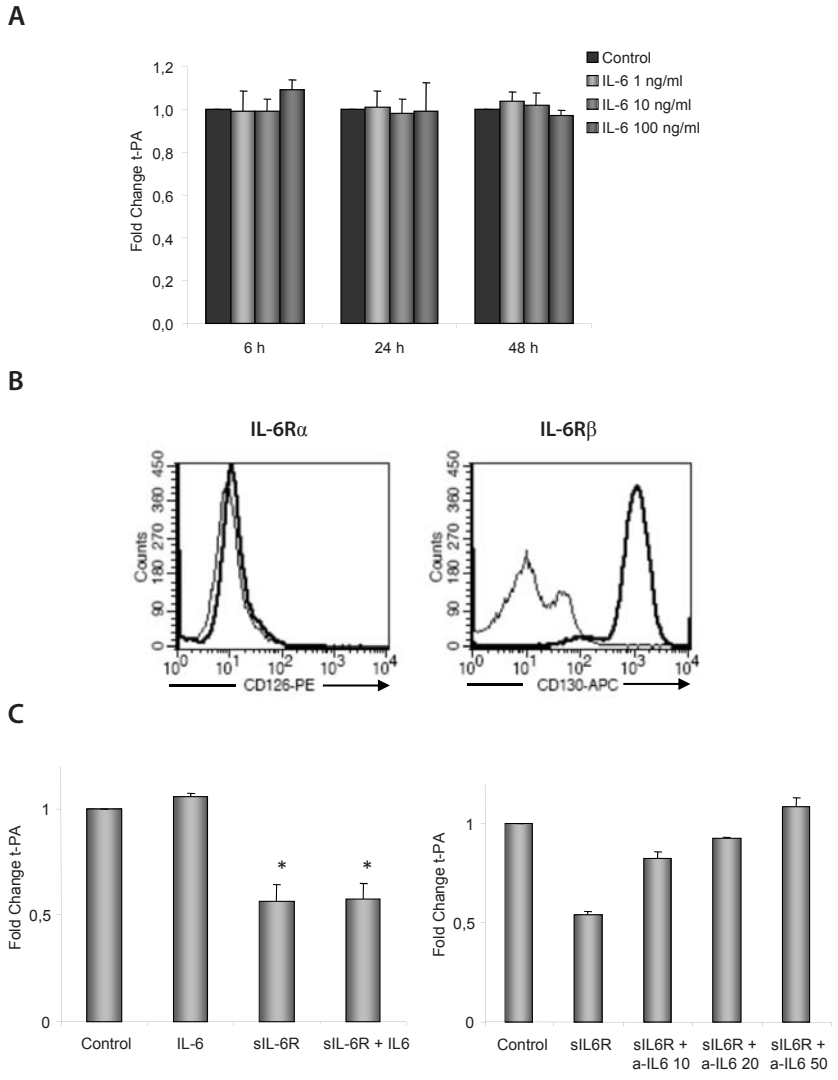


Figure 6. Effect of IL-6 stimulation on t-PA mRNA expression. **A**. HUVECs were exposed to 1-100 ng/ml of IL-6 for 6-48 h and mRNA analyzed. *n*=3. **B**. The surface expression of IL-6 receptor components CD126 (IL-6Rα) and CD130 (IL-6Rβ) was analyzed by flow cytometry. Data are representative of three individual experiments. The thin line represents isotype control antibody. **C**. Soluble IL-6 receptor α (sIL-6Rα) and neutralizing anti-IL-6 antibody (a-IL6) were included in the cultures for 24 h and t-PA mRNA quantified. *n*=2-3.

tect any effect of IL-6 on t-PA mRNA or protein production in HUVEC at any of the time points studied (Figure 6A), consistent with the one previous report on effects of IL-6 on t-PA [138]. Further experiments indicated that IL-6 did not affect expression of PAI-1, nor the positive control genes ICAM-1 or VCAM-1 (data not shown) indicating that HUVECs in this culture system may not be able to respond to this cytokine, i.e. may not express the IL-6

receptor. Examining the surface expression of the two IL-6 receptor components IL-6Rα and IL-6Rβ by flow cytometry, we found that whereas the absolute majority of HUVEC stained positive for the signal transducing IL-6Rβ we were unable to detect expression of the ligand-binding receptor subunit IL-6Rα (Figure 6B) indicating that the cells under these *in vitro* culturing conditions indeed are unable to respond to this cytokine. There is, however, a soluble form of IL-6Rα (sIL-6Rα) present in the circulation *in vivo* and this soluble receptor component has been described to confer IL-6 responsiveness to cells expressing IL-6Rβ but lacking endogenous IL-6Rα expression [142]. We thus exposed the HUVECs to recombinant sIL-6Rα either alone or in combination with IL-6. Addition of sIL-6Rα caused t-PA mRNA expression to fall by approximately 40 % even in the absence of exogenous IL-6 (Figure 6C). Addition of recombinant IL-6 to these cultures did not cause a further reduction of t-PA expression, indicating that receptor-saturating concentrations of IL-6 were already present in the cultures. Measurement of IL-6 levels in HUVEC conditioned media showed significant IL-6 production by these cells. In line with this, when adding increasing amounts of a neutralizing IL-6 antibody the reduction of t-PA mRNA caused by sIL-6R was completely abolished (Figure 6C).

Some previous reports have found cultured endothelial cells to be directly responsive to IL-6 [143,144,145], whereas others have reported that exogenous IL-6Rα is required to elicit an IL-6 response [146,147]. These discrepancies could possibly be due to differences in cell origin or culturing conditions. For example, it is possible that endothelial cells from different vascular beds have a differential expression pattern of IL-6Rα.

In summary, findings 1 and 2 show that prolonged exposure of endothelial cells to the pro-inflammatory cytokines TNF-α and IL-1β reduces the expression of t-PA, and that IL-6, in combination with its soluble receptor, also works as a suppressor of t-PA production.

Finding 3: NFκB signaling is central for cytokine suppression of t-PA, but p38 MAPK signaling can also be of importance

After determining the effects of pro-inflammatory cytokines on t-PA expression, we wanted to study the intracellular signaling mechanisms of importance for mediating the inflammatory signal causing the suppression of t-PA production. It has been reported in the literature that the NF-κB, p38 MAPK, and JNK pathways are activated by TNF-α in endothelial cells [136,148]. Using EMSA and phospho-specific western blot analysis after 1, 3, 6, or 24 h of cytokine stimulation we could confirm that TNF-α and IL-1β activates NF-κB, JNK and p38 MAPK signaling, most evident at the shorter time points (peak activation 1-6 h), and that after 24 h of cytokine stimulation very little increase in signaling activity remained. The kinetics of the activation was somewhat different between TNF-α and IL-1β mainly for JNK which reached maximal activation at the 1 h time point for IL-1β in contrast to the 6 h time point for TNF-α. We also investigated a potential ERK1/2 activation by IL-1β [149], but did not detect an obvious ERK1/2 activation during the time span examined. However, given that MAPK signaling can be very rapid and transient, it is possible that we overlooked an ERK1/2 activation by using 1 h as the first time point for analysis.

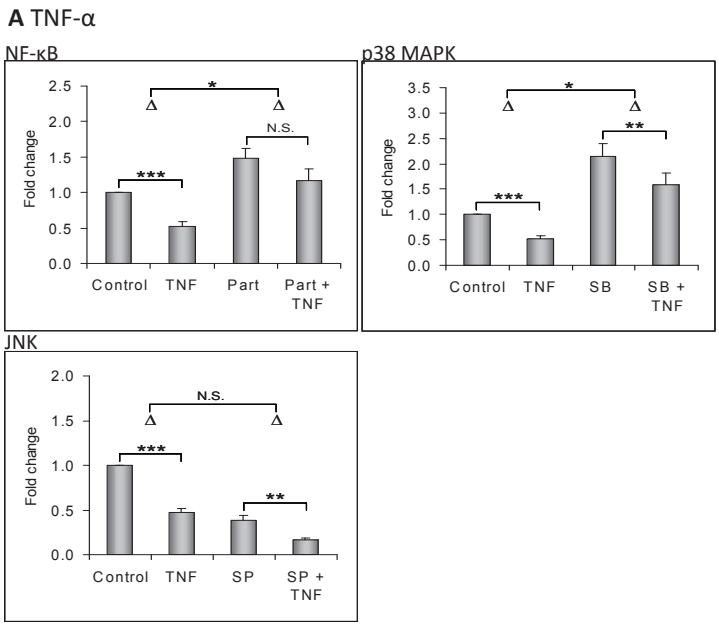
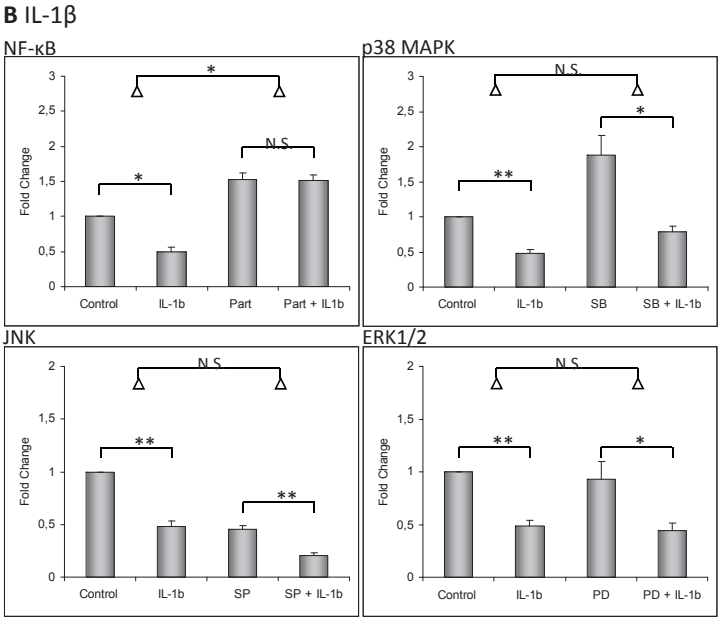


Figure 7. Effect of signaling pathway inhibition on t-PA mRNA expression. **A**. TNF-α signaling. *n*=6. **B**. IL-1β signaling. *n*=4. Cells were incubated with optimal concentration of pharmacological inhibitors blocking the NF-κB, p38 MAPK, JNK, or ERK1/2 pathways one hour before treatment with 1 ng/ml TNF-α or 10 pg/ml IL-1β for 24 h. t-PA mRNA was then quantified by real-time PCR. Relevant statistic comparisons are pointed out in the figures. Δ indicates relative difference between cytokine-stimulated cells and respective non-cytokine-stimulated controls. *p<0.05, **p<0.01, ***p<0,001



To investigate which of these activated signaling pathways that were directly involved in the cytokine induced t-PA gene suppression, HUVECs were treated with selective pharmacologic inhibitors of the signaling pathways before stimulation with TNF-α (Figure 7A) or IL-1β (Figure 7B). Blocking NF-κB and p38MAPK signaling, using parthenolide and SB203580, respectively, modulated the response to TNF-α whereas for IL-1β signaling, p38 MAPK was found to be of less importance. Furthermore, JNK and ERK signaling seemed to be of less importance for mediating the cytokine effect on t-PA. Hence these studies indicated that the suppression of t-PA in response to pro-inflammatory cytokines is preferentially an effect of NFκB signaling, but also pointed out a role for p38 MAPK signaling in TNF-α, but not IL-1β, induced suppression of t-PA. Signaling crosstalk between the NF-κB pathway and other intracellular signaling pathways, including the p38 MAPK pathway, has been described for other genes (reviewed in [150]). The physical accessibility of κB sites in the genome has been reported to vary as certain promoters contain κB sites of immediate accessibility, whereas in others, (e.g. the IL-6 and IL-12 promoters) the chromatin has to be structurally modified in order to expose the κB binding site [151,152]. p38 MAPK activation has been reported to affect histone modification status by increasing phosphorylation and acetylation of Histone H3, loosening the local chromatin structure around a number of κB sites [152]. Thus, p38 MAPK activity might be required for the κB factors to gain full access to the t-PA regulatory region. Why this would potentially be of importance for NF-κB dimers activated by TNF-α and not by IL-1β is not clear.

We then tried to reveal which down-stream effectors of NF-κB and p38 signaling that could be of importance for the t-PA suppression. NF-κB signaling in endothelial cells has been associated with activation of the NF-κB family transcription factors p65, p50 and c-rel, whereas p38 MAPK signaling mainly results in activation of transcription factors of the AP-1 family [136,153]. EMSA experiments using probes containing the t-PA specific κB element showed increased binding of nuclear proteins from TNF-α stimulated cells (Figure 8A). The binding patterns showed strongly induced binding at both 3 and 24 h. Supershift experiments identified the major cytokine induced shift (complex 1) to represent binding of the NF-κB subunits p50 and p65, indicating a p65/p50 heterodimer. For IL-1β a similar nuclear protein binding pattern was observed in EMSA experiments directed against the t-PA specific κB element (Figure 8B). What was more obvious in these experiments was the increase in binding of a complex identified by supershift analysis to contain p50 but not p65 or c-rel (complex 2 in Figure 8B). This could potentially represent a suppressive p50/p50 homodimer. TNF-α was observed also to increase binding to the t-PA specific, and AP-1 like, CRE element (data not shown). These experiments indicated activation of AP-1 by TNF-α and a potential enhanced binding of proteins from the AP-1 family to the t-PA CRE site. Instead, however, supershift experiments identified the TNF-α induced shift to represent binding of CREB. Transcription factors of the CREB/ATF family and the AP-1 family can dimerize and form heterodimers [154]. CREB has previously been demonstrated to be a part of dimers binding the t-PACRE in endothelial cells [104].

NF-κB dependent signaling has traditionally been considered to primarily mediate transcriptional up-regulation. The mechanism for a possible repressive function of NF-κB on t-PA gene expression is yet to be determined. Speculating about potential mechanisms, there are studies showing that NF-κB can function as a transcriptional repressor of other hemostatic

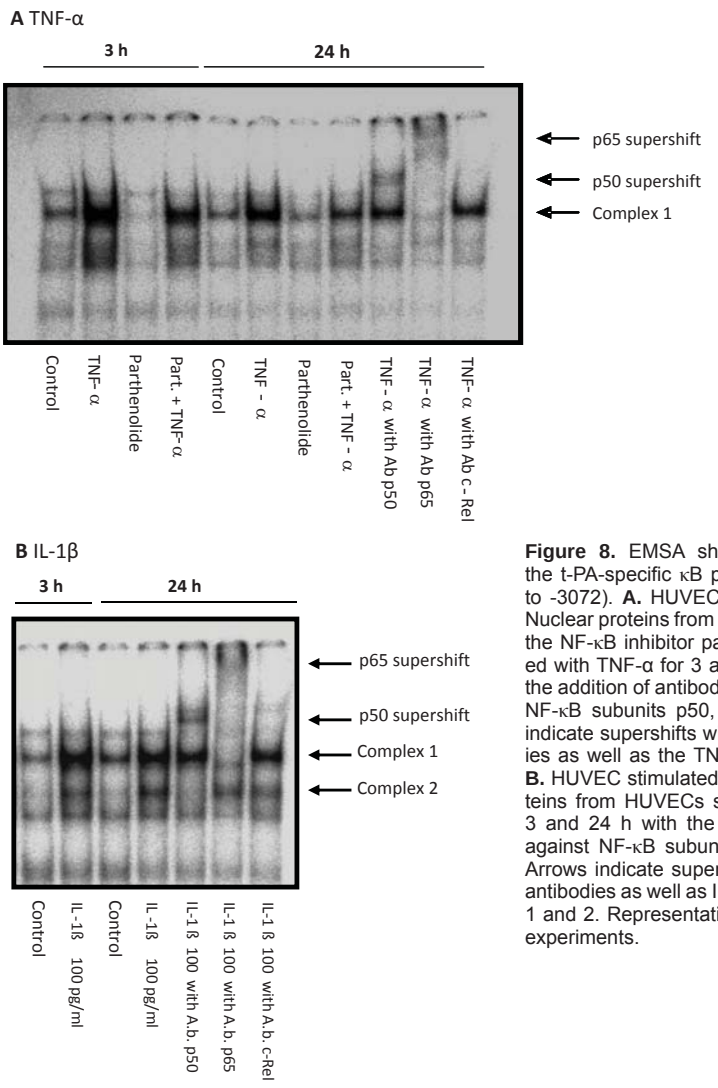


Figure 8. EMSA showing interactions with the t-PA-specific κB probe (position bp -3081 to -3072). **A.** HUVEC stimulated with TNF-α. Nuclear proteins from HUVECs pretreated with the NF-κB inhibitor parthenolide and stimulated with TNF-α for 3 and 24 h. Lane 9-11 with the addition of antibodies (Ab) 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 1. **B.** HUVEC stimulated with IL-1β. Nuclear proteins from HUVECs stimulated with IL-1β for 3 and 24 h with the addition of Ab directed against NF-κB subunits p50, p65 and c-Rel. Arrows indicate supershifts with p50 and p65 antibodies as well as IL-1β-induced complexes 1 and 2. Representative of three independent experiments.

molecules in endothelial cells [155,156]. 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 [157]. Speculating that the κB element is functional in HUVEC, this mechanism would be a tempting explanation as we found that an IL-1β stimulated EMSA shift, probably containing p50 homodimer, was induced at 24 h. In the last decade, NF-κB action has been increasingly recognized to be regulated by differential co-factor association (reviewed in [150]). Although traditionally considered to be associated mainly with coactivators, this transcription factor has lately been described to also function as a repressor by interacting with HDACs [158,159,160,161,162,163,164,165]. As such, a second potential mechanism by which NF-κB activation could reduce t-PA gene expression

is by interfering with HATs (such as p300 and CREB-binding protein) or recruiting HDACs to the t-PA promoter, reducing histone acetylation and as such making the local chromatin structure less permissive for the transcription machinery. This mechanism has for example been implied in the NF-κB mediated negative regulation of the thrombomodulin gene [156] and the MEF2 gene [160] in endothelial cells. Given the data presented in Study III and IV, suggesting that the t-PA gene is sensitive to histone acetylation status, these mechanisms are not unlikely to play a role in NF-κB mediated repression of t-PA transcription.

In summary, these studies show that pro-inflammatory cytokine-induced suppression of t-PA is mediated mainly by NF-κB signaling but p38 MAPK signaling is also of importance for the TNF-α, but not the IL-1β, effect. Activation of these signaling pathways increases binding of p65 and p50 to a probe containing the t-PA κB site as well as of CREB to the t-PA CRE site, potentially indicating a role for these elements in cytokine mediated t-PA suppression.

Study III and IV - Role of histone deacetylase inhibitors as stimulators of t-PA production

Besides inflammatory conditions, other risk factors including smoking, hypertension, and genetic background could also contribute to a low t-PA production. We hypothesize that restoring t-PA production, and thus the capacity for t-PA release, in such conditions may constitute a new potential target for prevention of atherothrombotic events. In search for a pharmacological tool that could be used to increase t-PA production in endothelial cells in man, irrespective of mechanism of suppression, we turned our attention towards substances with HDAC-inhibitory activity as the t-PA gene previously has been shown to be sensitive to changes in histone acetylation status [124,125]. Of considerable interest, there are a number of HDACis that are already in or close to clinical use. The majority of these belong to one of four chemically distinct classes: the short chain fatty acids (SCFA), the hydroxamic acids, the benzamides, or the cyclic peptides (reviewed in [166,167]). It is possible that various HDACi drugs have substantially different impacts on gene expression due to their differences in structure and HDAC-subclass targeting. In Study III, we therefore investigated a selection of clinically relevant HDACi substances to determine and compare their effect on the production of t-PA in cultured endothelial cells. In Study IV we more closely examined the role of the HDAC-inhibitory function of the anti-convulsant drug valproic acid (VPA) in the induction of t-PA expression.

Finding 4: Clinically used HDACis are potent stimulators of t-PA expression in endothelial cells

HUVECs were exposed to 11 different HDACis, representing the four major structural classes (Study III). Considering the differences in structure and HDAC enzyme-subclass targeting [167] we were surprised to find that all HDACis had similar effects on t-PA mRNA production. All HDACis tested increased t-PA mRNA levels after 24 h of culture but with different potencies (Figure 9 and Table 1). The least potent substances were the "old-generation" SCFA phenylbutyrate (PBA) and valproic acid (VPA) which both affected t-PA in the low

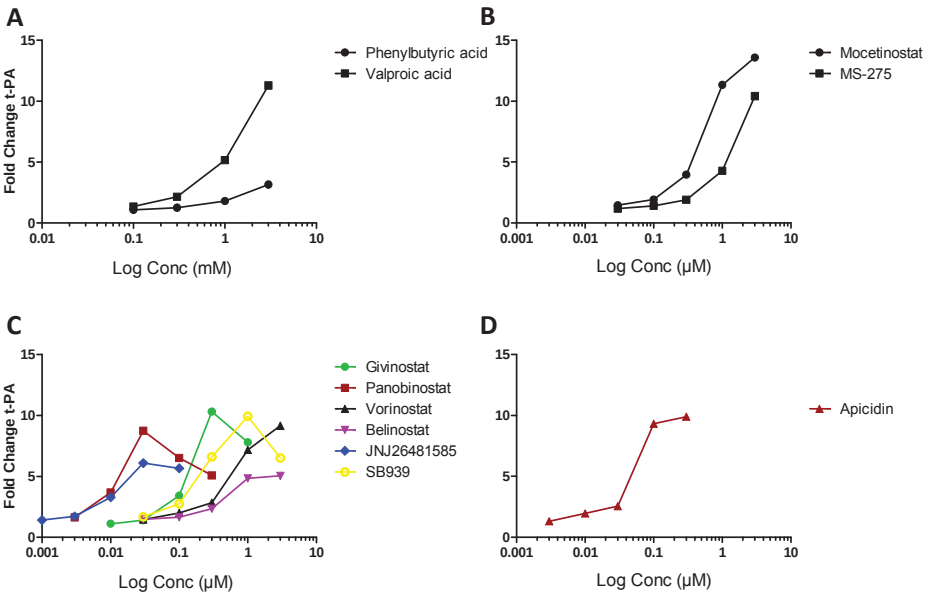


Figure 9. Effects of HDACis on mRNA expression in endothelial cells**.** HUVECs were exposed to different concentrations of HDACis for 24 h and mRNA was quantified by real-time PCR. Values are expressed as fold change over control cells. **A**. Short chain fatty acids. **B**. Benzamides. **C**. Hydroxamic acids. **D**. Cyclic peptide. *n*=3.

Table I. Summary of interesting dose-response observations. E_{max} is the maximal effect on t-PA in the dose-range tested. Conc for E_{max} is the concentration of HDACi at which the maximum effect occurred. Conc (100 % increase) is the concentration of each inhibitor that yielded a doubling of t-PA mRNA production and C_{max} is the maximal plasma concentration achieved in anti-seizure/oncology treatment in man.

Substance	E_{Max} (fold change)	Concentration for E_{max}	Concentration (100% increase)	C_{max} *in vivo*
SCFA				
Phenylbutyric acid	3.16	3 mM	1.3 mM	1 mM
Valproic acid	11.28	3 mM	0.3 mM	1 mM
Benzamides				
Mocetinostat	13.57	3 µM	0.1 µM	0.4 µM
MS-275	10.41	3 µM	0.3 µM	0.1 µM
Hydroxamic acids				
Belinostat	5.04	3 µM	0.2 µM	2 µM
Vorinostat	9.16	3 µM	0.1 µM	1 µM
SB939	9.93	1 µM	0.05 µM	0.6 µM
Givinostat	10.32	0.3 µM	0.05 µM	0.5 µM*
Panobinostat	8.74	0.03 µM	0.004 µM	0.06µM
JNJ26481585	6.09	0.03 µM	0.004 µM	N.A.
Cyclic Peptides				
Apicidin	9.90	0.3 µM	0.01 µM	N.A.

* Used for anti-inflammatory treatment

mM range. Out of these two, VPA appeared to be the more effective as PBA 3 mM only caused a 3-fold induction of t-PA mRNA whereas VPA of the same concentration gave an 11-fold increase. The concentrations needed to get a 2-fold induction of t-PA (100% increase, here arbitrarily defined as the C_{100}-value) were approximately 1.3 and 0.3 mM, respectively. In Study IV, we confirmed that the effect of VPA on t-PA was present also in the more relevant cell types, HCAEC and HAEC (data not shown), and showed that the dose-range was approximately the same as in HUVECs indicating that HUVEC is a relevant model cell type for these *in vitro* experiments. The two benzamides Entinostat and Mocetinostat reached comparable E_{max} (10-15 fold), but Mocetinostat appeared to be somewhat more potent with a steeper dose-response curve and a somewhat lower C_{100} value (approximately 300 and 100 nM, respectively). All hydroxamic acids had similar E_{max} (5-10 fold increase) but at different concentrations. Vorinostat and Belinostat were the least potent with C_{100} values of approximately 100 and 200 nM respectively. Givinostat and SB939 were somewhat more potent with C_{100} values of approximately 50 nM. The most potent substances were Panobinostat and JNJ26481585 with C_{100} values of approximately 4 nM. The cyclic peptide Apicidin had similar E_{max} as the other HDACis (10 fold) and a C_{100} value of approximately 10 nM.

To confirm that the effect seen on the level of mRNA was also translated to the protein level, we investigated levels of t-PA protein after 24 h of stimulation. The stimulating effect of HDAC inhibition on t-PA was, with the exception of Mocetinostat, also evident on the protein level (data not shown). For Mocetinostat this lack of protein induction after 24 h could perhaps be an effect of a slower kinetic profile as the results from a time series showed markedly higher protein secretion at later time points for this specific substance (data not shown). The time series further showed that for the majority of the HDACis studied, the increase in protein production was maintained for up to 72 h indicating that the effect on t-PA production is sustained (data not shown).

Of note, the HDACis were able to restore endothelial cell t-PA production when it had been suppressed by TNF-α (Figure 10). Pre-treatment with TNF-α decreased the expression of t-PA by approximately 50%, but after addition of low concentrations of the SCFA VPA, the hydroxamic acid Belinostat, or the benzamide Mocetinostat for 24 h, the t-PA levels were

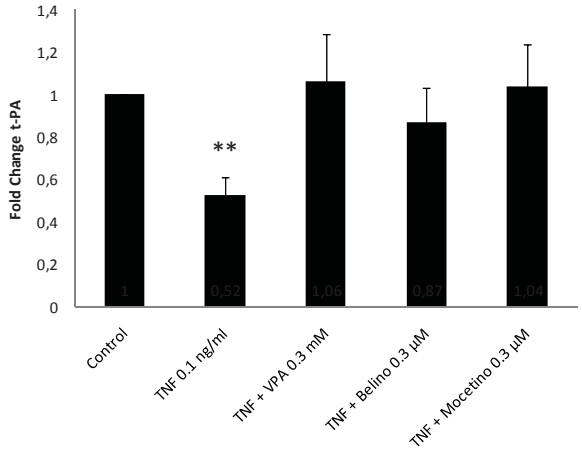


Figure 10. HDACis normalize t-PA expression in TNF-α treated cells. HUVECs were pre-treated with 0.1 ng/ml TNF-α for 24 h. Fresh TNF-α was then added together with 0.3 mM VPA, 0.3 µM Belinostat (Belino), or 0.3 µM Mocetinostat (Mocetino) and incubated for an additional 24 h. mRNA was quantified. *n*=4. **p<0.01.

normalized. The mechanism behind this normalization has not yet been determined and one can only speculate about the means by which HDACis can counteract the effect of TNF-α. The two mechanisms could be parallel and completely unrelated, the respective effects simply neutralizing one another. It could, however, also be that the HDACis directly affect post translational acetylation of the NFκB subunits, or inhibit suppressive HDACs recruited by NFκB to the t-PA promoter.

Finding 5: The HDAC inhibitory activity of valproic acid appears to be important for the induction of t-PA

The fact that all eleven HDACi substances tested, irrespective of chemical structure, stimulated t-PA production strongly suggests that it is indeed their HDAC-inhibitory function that targets t-PA. Moreover, the relative potencies of the substances in inducing t-PA correlates well with the relative IC_{50} values reported for class I HDAC enzymes (JNJ26481585 ≈ panobinostat > Apicidin ≈ Givinostat ≈ SB939 ≈ Mocetinostat ≈ Vorinostat ≈ Belinostat > Entinostat >>> VPA ≥ PBA) [168,169], supporting an HDAC inhibition effect. In Study IV, we further investigated if VPA, which despite long clinical use was only recently described to have HDAC-inhibitory function [170,171], seemed to stimulate t-PA *via* its ability to inhibit HDAC enzymes.

We compared the effect of VPA (2-propylpentanoic acid) on t-PA expression to that of valpromide (2-propylpentanamide), a structural amide analogue of VPA reported to lack HDAC inhibitory activity [170,172]. Whereas maximal concentrations of VPA consistently increased t-PA levels about 10-fold, no effect of the same concentration of valpromide on expression of t-PA mRNA in HUVEC could be detected (data not shown). To confirm that VPA functions as an HDACi in endothelial cells we investigated if VPA affected histone acetylation status in HUVEC both globally and also specifically at the t-PA transcription start site. Western blot assays with antibodies to acetylated and total histone H3 and H4 showed an increase of global acetylated histone H3 and H4 after VPA treatment (Figure 11A), confirming its HDAC-inhibitory activity in HUVEC. To investigate if VPA increases histone acetylation also at the t-PA gene regulatory region, ChIP-analyses were performed using antibodies to pan-acetylated histone H3 and H4, and primers flanking the major t-PA transcription initiation site. This showed a significant 2-fold increase of both acetylated H3 and H4 associated with the region surrounding the t-PA transcription start site after VPA treatment (Figure 11B). This increased acetylation of t-PA-associated histones was not a consequence of stimulated gene expression *per se* as HUVECs treated with simvastatin, a non-HDACi substance stimulating t-PA expression [130,173], did not result in increased histone acetylation surrounding the t-PA TIS (data not shown).

Accumulating evidence points to the existence of a histone code that is recognized and interpreted by effector proteins with chromatin modifying activities [119]. There are also data implying that certain specific modifications directly influence higher-order chromatin structure and compaction [174,175]. Thus, to confirm the results from the pan-acetyl antibody ChIPs and to get a more detailed view of the acetylation code surrounding the t-PA tran-

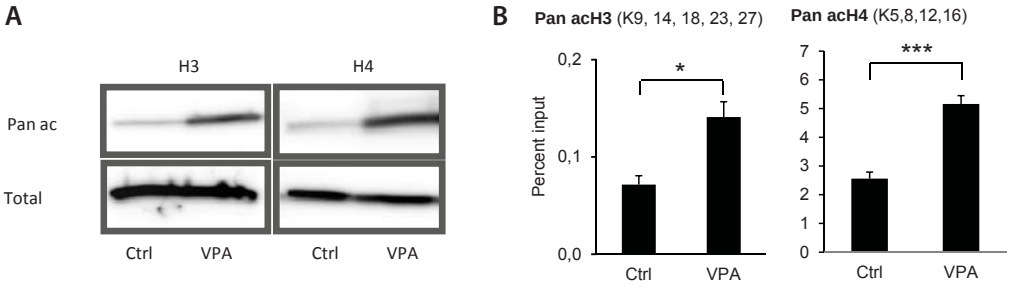


Figure 11. Effect of VPA on histone acetylation in endothelial cells. **A.** HUVECs were exposed to VPA for 24 h and global histone acetylation was assessed by western blot. **B.** ChIP. HUVECs were exposed to VPA for 24 h and acetylation of histones associated with the t-PA promoter was assessed by ChIP. Quantification of precipitated DNA was performed by real-time PCR. Data are presented as percent recovered DNA after the specific IP compared to non-precipitated input DNA, corrected for background binding (percent input). *n*=4-5
*p<0.05, **p<0.01, *** p<0.001.

scription start site, individual lysine acetylation in this region was investigated using ChIP with monolysine-specific antibodies. These ChIP assays demonstrated a significant increase of acetylation of lysines 9, 18, 23 and 27 on histone H3 (Figure 12A), as well as lysines 8 and 16 (but not lysines 5 and 12) on histone H4 (Figure 12B). Acetylation of H4K16 specifically has been reported to have a strong influence on higher-order chromatin structure [175]. Interestingly, this was one of the histone acetylation marks at the t-PA promoter that was markedly induced after VPA stimulation, perhaps indicating that compaction of chromatin in this area might be reduced by VPA treatment.

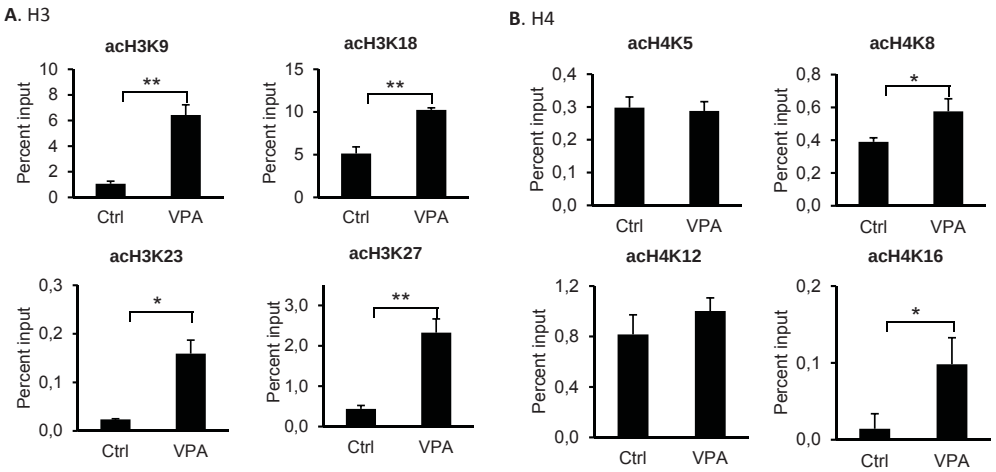


Figure 12. Effect of VPA on lysine marks in the t-PA promoter. HUVECs were exposed to VPA for 24 h after which cells were fixed and chromatin harvested. **A.** ChIP for specific histone H3 mono-lysine acetylation. Antibodies for monoacetylated acH3K9, acH3K14, acH3K18, acH3K23 and acH3K27 were used. *n*=4. **B.** ChIP for specific histone H4 mono-lysine acetylation. Antibodies for monoacetylated acH4K5, acH4K8, acH4K12 and acH4K16 were used. *n*=5. Quantification was performed with real-time PCR primers flanking the t-PA transcription start site. Data are presented as percent recovered DNA after the specific IP compared to non-precipitated input DNA, corrected for background binding (percent input).
*p<0.05, **p<0.01, *** p<0.001.

In an attempt to identify which specific HDAC enzyme(s) that are involved in the regulation of t-PA, we used siRNA-mediated knock-downs of class I HDACs and determined the effect of selective depletion of individual HDACs on the response to VPA (Figure 13). Treatment of cells with HDAC1 or HDAC2 siRNA had no effect on basal t-PA mRNA expression. However, a small, though significant, induction of t-PA mRNA was observed in HDAC3 and HDAC8 depleted cells compared to untreated controls perhaps indicating an importance for these enzymes in constitutive suppression of t-PA. In these siRNA experiments we were unable to detect a major involvement of any specific class I HDAC in the VPA-response, as the t-PA induction by VPA in each setup of HDAC1,2,3 or 8 siRNA treated cells was very similar to that in control cells. This lack of effect on the VPA response could potentially be due to possible redundancy among the class I HDACs or, alternatively, that HDACs of other classes (class II and IV) could be involved.

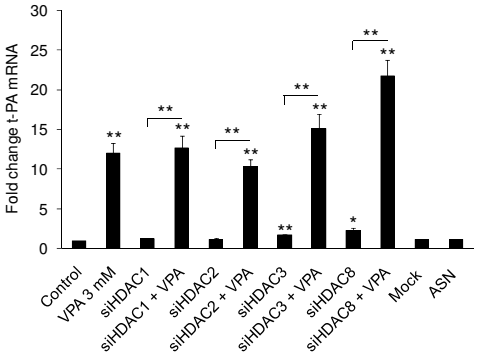


Figure 13. siRNA mediated knock-down of class I HDACs. t-PA mRNA expression in HUVECs treated with class I HDAC-targeting siRNAs for 72 h and then stimulated with 3 mM VPA for 24 h. *n*=4. Mock indicate cells treated with DharmaFECT 4 transfection reagent alone and ASN indicate cells transfected with All Star Negative control siRNA. Unless indicate in the figure, statistical comparisons are made relative to untreated control cells.
*p<0.05, **p<0.01.

Collectively, these results point towards a role for HDAC enzymes in the regulation of t-PA, and more specifically it appears that the t-PA gene is indeed constitutively suppressed by HDACs in endothelial cells, possibly by an epigenetic mechanism. This was also reported by the group of Kruithof who recently described increased t-PA expression and histone acetylation in a region approximately 1 kb upstream of the t-PA TIS after treatment with TSA, butyrate or Entinostat [117]. However, as HDACs also deacetylate many non-histone proteins, including transcription factors suggested to interact with the t-PA promoter, e.g. Sp1, Sp3, and NF-κB p65/p50 [176,177,178], it may not necessarily merely be a change in chromatin structure that causes the effect on t-PA. It is, of course, also possible that the effect is indirect *via* the HDACi-mediated increased production of e.g. a t-PA stimulatory factor.

Taken together findings 4 and 5 show that HDACis are potent regulators of endothelial t-PA production and support the hypothesis that it is indeed the HDAC inhibitory property per se that stimulates t-PA production, although the specific HDAC enzyme(s) regulating the t-PA gene remain(s) to be identified.

IMPLICATIONS AND POTENTIAL CLINICAL APPLICABILITY

The data presented in this thesis indicate that a pro-inflammatory environment is a potent suppressor of t-PA synthesis in cultured endothelial cells as both TNF-α and IL1-β turned out to be suppressors of t-PA production, and IL-6 was shown to have the potential for suppression in conditions where the intact IL-6 receptor is present. Furthermore, HDACis appear to be potent stimulators of t-PA gene expression in the same *in vitro* system. However, these findings are all based on *in vitro* models and it is not clear how these results translate to the complex *in vivo* situation and this clearly has to be examined in *in vivo* models. In the mean time, in the following section, I aim to relate these results to the *in vivo* situation and speculate about the potential relevance of the findings. More specifically, two major issues will be considered: 1. *What is the role of inflammation as a suppressor of t-PA in vivo?* and 2. *Could HDACis be potential candidates to stimulate t-PA production in man?*

What is the role of inflammation as a suppressor of t-PA *in vivo*?

Systemic circulating levels of TNF-α and IL-1β in the healthy state are reported to be <20 pg/ml for TNF-α and <5 pg/ml for IL-1β (reference values from the Sahlgrenska University hospital lab). In severe inflammatory autoimmune conditions such as in rheumatoid arthritis and vasculitides these cytokine levels can increase several-fold, especially for TNF-α, but are still often lower than the concentrations that we have used in our cell culture system. However, there are also local sites of inflammation in the vasculature with potentially high levels of inflammatory cytokines. One such local site of inflammation is the atherosclerotic plaque. During the last two decades atherosclerosis has progressively been recognized to be an inflammatory disease of the vessel wall (reviewed in [67,68]). Macrophages activated by the ingestion of oxidized lipids secrete a number of inflammatory substances including chemokines and pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and monocyte chemoattractant protein-1 (MCP-1) [141]. This in turn attracts other inflammatory mediators, including T-cells and mast cells, further escalating the inflammatory process by e.g. the secretion of more IL-6, as well as IL-2 and interferon-γ (IFN-γ) [141]. As a result, the vulnerable atherosclerotic plaque has been described to contain inflammatory reactions of equal intensity to that found in the synovium in acute rheumatoid arthritis [7]. Local levels of pro-inflammatory cytokines in and surrounding a plaque are difficult to estimate, but have in the case of TNF-α been measured to reach 0.2 ng/ml in carotid plaques [179]. Of interest, cells of the vascular wall, including endothelial cells and smooth muscle cells, are also able to produce cytokines when activated by a pro-inflammatory environment [141] and these could potentially contribute to locally elevated levels of inflammatory cytokines in a paracrine or autocrine fashion. Hence, it is conceivable that the endothelial cells surrounding the plaque are indeed exposed to a highly inflammatory environment with significant levels of TNF-α and/or IL-1β, either from the luminal side of the vessel, or likely also from across the basal membrane side facing the atheroma.

When exposed to TNF-α or in particular IL-1, vascular smooth muscle cells have been shown to produce very large amounts of the pro-inflammatory cytokine IL-6 [180] and IL-6 is also produced by cells in the atheroma including macrophages [141]. Our data indicate that, in addition to TNF-α and IL-1β, IL-6 has a potential suppressive effect on t-PA gene expression in endothelial cells, but that the effect is dependent upon whether or not an intact receptor signaling system is present in the particular vascular bed investigated. Specifically, it appears that IL-6 responsiveness requires either endogenous expression of surface bound IL-6Rα or that the soluble form of the receptor is provided by the cell's environment. Of considerable interest, it has been shown that neutrophils exposed to an inflammatory environment may shed IL-6Rα from their surface, a mechanism that may make the receptor available in tissues in which it is not normally expressed [146]. It was recently described that vulnerable plaques contain a considerable number of neutrophils [181,182] indicating that sIL6Rα may indeed be available in the local environment surrounding the plaque. Thus it is conceivable that the endothelial cells in this environment may be able to respond to the IL-6/sIL6R complex potentially present in the local area surrounding a plaque, further suppressing t-PA.

Interestingly, in contrast to the data presented in this thesis, *in vivo* studies on humans have shown substantial inductions of t-PA release when challenged with TNF-α [183,184,185,186]. In two reports from Newby's laboratory, the authors employed the perfused-forearm model to study acute effects of intraarterial TNF-α infusions either in healthy subjects [183] or in patients with stable coronary heart disease [185], and found that TNF-α has a stimulatory effect on t-PA release. Van Hinsbergh *et al.* [184] studied cancer patients that received a continuous intravenous infusion of TNF-α during 24 h. They reported elevated plasma concentrations of t-PA after 3 and 24 h of infusion, but speculated that this response may be secondary to a concomitant TNF-α induced generation of thrombin. Finally, van der Poll *et al.* [186] 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.

The acute nature of these *in vivo* studies means that they most likely examine the effect of TNF-α on t-PA release, not production. If the kinetics of t-PA suppression from our *in vitro* data can be extrapolated to the *in vivo* situation, then in order to see a decrease in local t-PA release the endothelium must be exposed to inflammatory cytokines for at least 24-48 h in order for t-PA gene expression to switch to a suppressive mode, and this reduction must then be transferred to the intracellular storage pools of t-PA. Therefore the effect of an extended, chronic inflammatory stimulus on t-PA release capacity needs to be examined *in vivo*. In this context, it is interesting to note that chronic low-grade inflammation has indeed been associated with a reduced capacity to release t-PA after venous occlusion of the forearm [187].

It is of note that although the cells of the plaque lesion, including smooth muscle cells and macrophages, often are described to produce high levels of t-PA [188,189,190,191], it has been shown that t-PA levels in macroscopically normal intima segments surrounding an atherosclerotic plaque are reduced in comparison with normal vessels [192]. Moreover, as mentioned previously, there is an inverse correlation between plaque burden and capacity for stimulated t-PA release in the coronary circulation [62]. This reduction of endothelial t-PA surrounding the plaque could be the consequence of several factors, including a disturbed

flow profile or general endothelial dysfunction, but it is also possible that it, at least in part, could be an effect of inflammatory cytokines suppressing t-PA production.

Taken together, the atherosclerotic plaque is an important site of local inflammation that contains substantial levels of TNF-α, IL-1β, IL-6 and potentially also sIL6Rα. This indicates that the local t-PA production in the endothelium surrounding the plaque could be significantly impaired, attenuating the local thrombotic defense where it is most needed. More data from a clinical chronic inflammatory in vivo setting are motivated to determine if this is indeed true in the in vivo situation.

Could HDACis be considered as stimulators of t-PA production in man?

It is evident that several factors, both of genetic and aquired nature, reduce the amounts of t-PA available for acute release, and that this is associated with increased risk of thrombotic events. So far, a means to pharmacologically stimulate endogenous t-PA production in man has been lacking. Could, then, the HDAC-inhibitory substances investigated in this thesis be considered as potential leads to stimulate a suboptimal t-PA production in man when suppressed by inflammatory or other patho-physiological stimuli?

The main advantage of these substances compared to previous leads is that they, to different extents, all have been administered to humans and hence clinical experience, pharmacokinetics and safety documentation in man already exist. The relative potency of these substances for t-PA induction also seems promising.

New generation HDACis in vivo

The new generation HDACis have mainly been developed for use in oncology applications as these substances have been shown to have anti-tumor effects, possibly by reactivating epigenetically silenced tumor suppressor genes [193]. In doses used for oncology treatment, however, all new-generation HDACis are associated with adverse effects, including nausea, fatigue, headache, thrombocytopenia, and for certain substances also EKG-abnormalities and liver damage that clearly would limit their use in cardiovascular prevention. Interestingly, however, our data show that, at least for members of the hydroxamic acid class, the concentrations needed to stimulate t-PA *in vitro* (C_{100}) are generally approximately 10-fold lower than the concentrations used to obtain antitumor effects *in vivo* or *in vitro* [194,195]. Given that the concentrations are transferable *in vivo* and *in vitro* in other models [194,195], this indicates that significantly lower doses of the hydroxamic acids than those used for oncology treatment may be sufficient to stimulate t-PA *in vivo*. These lower doses would likely be associated with less risk for adverse events.

In support of this, safety and pharmacokinetics data for low doses of Givinostat on healthy subjects were recently reported [196]. The doses used in that study yielded plasma concentrations of approximately 100-200 nM with no adverse events reported. The C_{100} value for Givinostat in our system was around 50 nM, i.e. even lower than in the above mentioned study. Taken together, if assuming *in vitro-in vivo* transferability, the low concentrations of HDACi needed to increase t-PA imply that the side effect profile for the hydroxamic acids may still be favorable even for use in cardiovascular prevention.

Valproic acid in vivo

The most extensively used HDAC-inhibitory substance in the clinic is the short chain fatty acid valproic acid. This substance has been used in the treatment of epilepsy and bipolar disorder for decades and thus extensive safety documentation for long-term treatment is available. The majority of side effects associated with VPA treatment are considered mild and reversible. The most serious adverse effects found in epileptics are liver damage, pancreatitis, teratogenicity and increased risk of bleeding [197]. However, these side effects are rare and most commonly occur in children and/or in the higher therapeutic range. In favor of a potential use in thrombosis prevention, we found that the concentration of VPA required to increase t-PA production by 100% (C_{100}) was 0.3 mM, which is just around the lower limit of the therapeutic plasma concentrations achieved *in vivo* (0.35-1 mM). If the t-PA stimulating effect seen *in vitro* occurs at the corresponding concentrations *in vivo*, these concentrations may be low enough to avoid the majority of the side effects associated with this drug.

Of considerable interest, and potentially supporting an effect of VPA on the fibrinolytic system also *in vivo*, VPA treatment in epilepsy has been associated with a protection against atherothrombotic events. In a recent retrospective pharmacoepidemiological study, Olesen *et al* compared the risk for myocardial infarction in epilepsy patients receiving valproate compared to age- and gender-matched controls from the general population. This study demonstrate that VPA treatment was associated with a significant 40 % risk reduction of myocardial infarction in Danish epilepsy patients on long term treatment with valproate [198]. This indicates that VPA has effects on atherothrombotic processes, potentially at least in part *via* activation of the fibrinolytic system.

Taken together, increasing endothelial t-PA production may be a novel target for the prevention of ischemic vascular disease but good pharmacological leads achieving this have been lacking. The data presented in this thesis indicate that HDACis could be promising candidates for such leads given their in vivo compatibility and apparent potency. However, even if they were to achieve an increased t-PA production in vivo, their safety profiles will be crucial as only minimal side effects will be tolerated.

SUMMARY AND CONCLUDING REMARKS

In summary, this thesis presents five main findings:

1. Extended exposure of cultured endothelial cells to the pro-inflammatory cytokines TNF-α or IL-1β suppresses t-PA production (Paper I and II).

2. The pro-inflammatory cytokine IL-6 has the capacity to suppress t-PA production if the IL-6 receptor component IL-6Rα is present (Paper II).

3. The TNF-α or IL-1β suppression of t-PA production is mainly mediated by NF-κB signaling (Paper I and II).

4. Substances inhibiting classical HDAC enzymes are potent stimulators of t-PA production in cultured endothelial cells (Paper III).

5. The HDAC-inhibitory activity of these substances is likely to be of importance for the stimulatory effect on t-PA (Paper III and IV).

The data and findings presented in this thesis clarify the role of pro-inflammatory cytokines in the production of the key intravascular thromboprotective initiator t-PA. The results point towards a suppressive role of inflammatory cytokines on the fibrinolytic system, not only by increasing PAI-1 as previously described, but also by a marked suppression of endothelial t-PA production. In the context of a vulnerable plaque, the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in combination with its soluble receptor, are present in high concentrations. It is conceivable that this inflammatory milieu could hamper the production of the clot-dissolving system locally, in the specific parts of the vasculature where it is most critical to have sufficient amounts of t-PA in the storage pools. This may contribute to the increased risk of cardiovascular disease that has been observed in patients with systemic markers of local inflammation, i.e. CRP.

However, the data presented in this thesis also open up an interesting new approach to normalize or boost the body's own protection system against occluding thrombosis when reduced by inflammatory or other patho-physiological stimuli. A means to pharmacologically stimulate endothelial t-PA production in high risk patients may represent a new preventive strategy for ischemic vascular disease. The HDAC inhibitors described in this thesis were found to be potent and selective stimulators of t-PA, and could as such represent the first possibility of actually testing this approach in man.

These studies are, however, all based on results generated in *in vitro* cell culture models. Although highly applicable for the type of studies performed in this thesis, it is still possible that the results are not transferable to the complex *in vivo* situation. Therefore, the results now need to be confirmed in *in vivo* models.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hjärtinfarkt och instabil kärlkramp orsakas vanligen av att en blodpropp plötsligt bildas i ett av de blodkärl som försörjer hjärtmuskeln med syre och näringsämnen. Detta leder till att en svår syrebrist uppstår i hjärtat, som hotar att på kort tid ge en obotlig skada av hjärtmuskeln. Skadan kan ge livshotande störningar i hjärtrytmen eller allvarligt försämra hjärtats pumpförmåga. För att förhindra detta har kroppen ett inneboende försvarssystem mot blodproppsbildning, det fibrinolytiska systemet, som bryter ner blodproppen och hindrar att den växer okontrollerat och täpper igen kärlet. Man uppskattar att en tredjedel av hotande hjärtinfarkter kan undvikas genom att kroppen själv löser upp blodproppen. Troligen sker proppbildning ganska ofta utan att ge några symtom tack vare att kroppen hinner lösa upp propparna innan syrebrist och smärta uppstår. En nyckelspelare i aktiveringen av detta blodproppsupplösande system är ett enzym, vävnads-plasminogenaktivator, förkortat t-PA, som frisätts från innersta cellagret i kärlväggen då en propp börjar bildas. Det är viktigt att ha tillräckligt stora depåer av t-PA upplagrade i kärlväggen för att få ett effektivt försvar, och risken för hjärtinfarkt ökar om depåerna är mindre. Därför är det oroande att t-PA depåerna kan reduceras av olika faktorer, både ärftliga och livsstilsberoende. Exempelvis har högt blodtryck, rökning och övervikt visat sig minska dessa depåer, något som förmodligen bidrar till den ökade risken för hjärtinfarkt vid dessa tillstånd.

Inflammation är ett annat tillstånd som skulle kunna påverka t-PA depåerna. Vid inflammation släpper kroppens immunsystem ut en mängd faktorer som styr den inflammatoriska processen. Dessa faktorer kallas cytokiner och innehåller bland annat TNF-α, IL-1β, och IL-6. I den första delen av avhandlingsarbetet så undersöktes hur dessa inflammationsfaktorer påverkar produktionen av t-PA i odlade celler från kärlväggen. Vi fann att både TNF-α och IL-1β hade en direkt hämmande effekt på t-PA produktionen, men att det krävs ganska lång tid (ungefär ett dygn) för effekten att uppstå, vilket kan vara en anledning till att resultaten från tidigare studier har varierat. Vi kartlade även de signaleringsvägar som överför den hämmande inflammationssignalen från cellens yta till genen som kodar för t-PA proteinet. Den tredje inflammationssubstansen, IL-6, hade också en hämmande effekt på t-PA produktionen, men bara om en viss receptorkomponent som cellerna själva inte kan producera fanns närvarande. Vid åderförkalkning i kärlväggen, den absolut vanligaste orsaken till att blodproppar bildas, så finns alla dessa inflammationsfaktorer närvarande i höga koncentrationer och skulle alltså kunna minska t-PA depåerna. Det är därför troligt att just lokalt i den miljön där t-PA depåerna är som viktigast, så är också produktionen av detta ämne hämmat av inflammation.

Eftersom t-PA verkar vara viktigt för att motverka blodproppsbildning inuti kärlen så skulle ett läkemedel som ökar t-PA depåerna kunna hjälpa kroppen att bli bättre på att själv förebygga hjärtinfarkter. Det har dock varit svårt att hitta kemiska ämnen som kan stimulera produktionen av t-PA i människa. De flesta substanser man provat har antingen visat sig ha för mycket biverkningar eller inte varit lämpliga att ge som läkemedel. I den andra delen av avhandlingsarbetet undersöktes om en viss molekylklass, så kallad HDAC-inhibitorer, stimulerar t-PA produktionen i odlade celler från kärlväggen. De substanser som undersöktes används redan idag som läkemedel för andra sjukdomar, bl.a. epilepsi och cancer,

vilket innebär att deras läkemedelsegenskaper och biverkningar är kända. Vi fann att samtliga HDAC-inhibitorer ökade t-PA produktionen i våra odlade celler och att vissa av dessa verkade åstadkomma detta vid mycket lägre koncentrationer än förväntat. De kunde även återställa t-PA produktionen då denna var reducerad av inflammation.

Sammanfattningsvis så visar resultaten av denna avhandling att faktorer närvarande vid inflammation hämmar t-PA produktionen, men att kliniskt använda HDAC-inhibitorer kan stimulera/återställa produktionen. Det är vikigt att ha i åtanke att hittills är alla försök utförda i odlade celler. Trots att detta är ett bra modellsystem för denna typ av studier så skiljer sig den miljö som cellerna befinner sig i markant från deras normala tillvaro i kroppen. Därför kan man inte garantera att cellerna beter sig på samma sätt när de är i sin naturliga miljö i kroppen och dessa resultat måste därför verifieras i människa. Nyligen visade dock en studie av en grupp i Danmark [198] att den mest kliniskt använda av de undersökta HDAC-inhibitorerna har en skyddande effekt mot hjärtinfarkt när den ges som epilepsibehandling. Det är tänkbart att i alla fall delar av detta skydd skulle kunna bero på en ökning av t-PA depåerna i kärlväggen så att kroppen får ett bättre försvar när en blodpropp väl har börjat bildas.

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