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Endogenous t-PA release and pharmacological thrombolysis

Experimental animal studies of the coronary circulation

Jan-Arne Björkman

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Endogenous t-PA release and pharmacological thrombolysis
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Abstract

The physiologically most important activator of intravascular fibrinolysis is tissue-type plasminogen activator (t-PA) released from endothelial cells. In man, sympathomimetic drugs increase the systemic concentration of t-PA. It is therefore of interest to investigate whether cardiac sympathetic activation can induce a local t-PA release, which could counteract intra-coronary clot formation.

Thrombolytic therapy with recombinant t-PA (rt-PA) is effective in acute myocardial infarction, but the treatment is limited by a fairly slow reperfusion rate and frequent early reocclusions. A potential mechanism behind early reocclusions might be that active thrombin is released from the thrombus during thrombolytic therapy. Thrombin has recently been shown to activate pro-carboxypeptidase U, which in its active form (CPU) down-regulates endogenous fibrinolysis. Therefore, one way of improving thrombolytic efficacy may be to combine rt-PA with a low-molecular weight direct thrombin inhibitor, which theoretically could have a pro-fibrinolytic effect, either by inhibition of fibrin-bound thrombin and/or by inhibition of CPU activation. An alternative way may be direct inhibition of CPU.

In a porcine model, experimental activation of cardiac sympathetic nerves by electrical stimulation at 1 and 8 Hz induced 5- and 20-fold increase in the release of both total and active t-PA together with frequency-dependent increases in heart rate, blood pressure, and coronary blood flow. The t-PA release was independent of the heart rate and coronary flow response, but local infusion of isoprenaline suggested that part of the t-PA response was mediated by stimulation of β-adrenergic receptors. Next, we studied the combined effect of rt-PA and thrombin inhibitors (melagatran, hirudin and heparin) in a canine model of copper coil-induced coronary thrombosis. The pro-fibrinolytic effect of rt-PA, either measured as patency rate or time-to-patency, was significantly enhanced with the low-molecular weight direct thrombin inhibitor melagatran, but to a lesser degree by hirudin and heparin. In the same model it was shown that active CPU is produced locally in the coronary vascular bed during both thrombus formation and clot lysis. Inhibition of thrombin attenuated CPU formation and improved patency. A similar effect was obtained with a direct inhibitor of CPU.

In conclusion, the coronary t-PA response to sympathetic stimulation may constitute a thrombo-protective defence mechanism to counteract its prothrombotic effects on the systemic level. Furthermore, direct thrombin and/or CPU inhibition may be potential targets for prevention of thrombus formation via facilitation of the endogenous fibrinolytic system.

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LIST OF ORIGINAL PAPERS

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

I. Björkman J-A, Jern S, Jern C**.** Cardiac sympathetic nerve stimulation triggers coronary t-PA release**.**
Arteriosclerosis, Thrombosis & Vascular Biology 2003;23(6):1091-7.

II. Mattsson C, Björkman J-A, Ulvinge JC. Melagatran, hirudin and heparin as adjuncts to tissue-type plasminogen activator in a canine model of coronary artery thrombolysis.
Fibrinolysis & Proteolysis 1997;11(3):121-8.

III. Mattsson C, Björkman J-A, Abrahamsson T, Nerme V, Schatteman K, Leurs J, Scharpe S, Hendriks D**.** Local proCPU (TAFI) activation during thrombolytic treatment in a dog model of coronary artery thrombosis can be inhibited with a direct, small molecule thrombin inhibitor (melagatran).
Thrombosis & Haemostasis 2002;87(4):557-62.

IV. Björkman J-A, Abrahamsson TI, Nerme VK, Mattsson CJ**.** Inhibition of carboxypeptidase U (TAFIa) activity improves rt-PA induced thrombolysis in a dog model of coronary artery thrombosis.
Thrombosis Research 2005;116(6):519-24.

CONTENTS

ABBREVIATIONS

ADP	adenosine diphosphate
ACS	acute coronary syndrome
APC	active protein C
APTT	activated partial thromboplastin time
ASA	acetylsalicylic acid
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
COX	cyclooxygenase
CPU	carboxypeptidase U = TAFIa
CPN	carboxypeptidase N
CVD	cardiovascular disease
DTI	direct thrombin inhibitors
DVT	deep vein thrombosis
EC	endothelial cell
GP	glycoprotein receptors
Hct	haematocrit
IPR	isoprenaline
LAD	left anterior descending coronary artery
LMWH	low-molecular-weight heparins
MAP	mean arterial blood pressure
MI	myocardial infarction
NO	nitric oxide
PAI-1	plasminogen activator inhibitor type-I
PAR	protease-activated receptor
PCI	percutaneous coronary intervention
PDE	phosphodiesterase
PE	phenylephrine
PGI_2	prostacyclin
PTCI	potato tuber carboxypeptidase inhibitor
rt-PA	recombinant tissue-type plasminogen activator
SNP	sodium nitroprusside
STEMI	myocardial infarction with ST elevation
TAFI	thrombin activatable fibrinolysis inhibitor = proCPU
TF	tissue factor
TXA_2	thromboxane A_2
t-PA	tissue-type plasminogen activator
UFH	unfractionated heparin
VKA	vitamin K antagonists
vWF	von Willebrand factor

INTRODUCTION

Cardiovascular disease (CVD) is currently the leading cause of death and illness in developed countries, and acute vascular syndromes are becoming a major concern worldwide [1]. The main manifestations of CVD are coronary artery disease (CAD), stroke and peripheral artery disease, among which CAD accounts for the highest mortality figures. The acute coronary syndromes (ACS), *i.e.* unstable angina and myocardial infarction (MI), are usually caused by an intraluminal thrombus in a coronary artery. There are several different mechanisms that can precipitate this thrombus formation [2]. One common cause is a disruption or tear in the cap of a lipid-rich atherosclerotic plaque in a coronary artery. This results in exposure of the lipid-rich atheromatous gruel to circulating blood, thus triggering local thrombus formation. A second cause is denudation or erosion of the endothelium with concomitant exposure of the subendothelial prothrombotic matrix. Other causes of coronary thrombosis are erosion of calcium nodules and intraplaque haemorrhage arising from the microvasculature of the plaque itself.

As a logical consequence of the fact that the dominant mechanism in ACS is thrombus formation, the main objective of treatment for ACS is to remove the thrombus and/or reduce the risk of it propagating into an occlusive thrombus. If the affected artery is already occluded, as in MI with ST elevation (STEMI), the primary aim is to restore blood flow either by pharmacological (*i.e.* thrombolysis) or interventional means (*i.e.* percutaneous coronary intervenetion (PCI) or coronary artery bypass grafting). On the other hand, if the affected artery is not occluded, efforts are directed towards preventing progress of thrombus formation by inhibiting platelet activation and aggregation and/or components of the coagulation cascade. Pharmacological means to achieve this are discussed below.

In the latter half of the previous century, coronary angiography was introduced permitting visualisation of coronary stenosis and occlusions in the living patient. Interestingly, investigations applying this technique have shown that, in approximately 30% of events with acute myocardial infarction, the infarction-related artery spontaneously reperfuses [3]. This demonstrates that the body has a potent endogenous defence mechanism to protect against formation of occluding arterial thrombi. The main activator of the fibrinolytic system in the intravascular compartment is tissue-type plasminogen activator (t-PA). In line with this, recombinant t-PA (rt-PA) is the most frequently used substance

for inducing thrombolysis by pharmacological means. Endogenously, t-PA is released from endothelial cells. As discussed below, triggers of this release include substances formed during activation of plasma coagulation and/or platelet, which makes this process an important feedback loop to counteract clot formation. This process can probably explain spontaneous reperfusion in acute MI. In line with this hypothesis, it has been found that subjects carrying a variant of the t-PA gene with a low capacity for t-PA release are at greater risk of suffering a MI [4,5]. This finding was recently reproduced in the Framingham cohort [6]. A reduced capacity to release t-PA has also been observed in subjects with an increased risk of myocardial infarction, *i.e.* smokers [7,8] and hypertensives [9,10]. It has, however, recently been shown, that the impairment in the latter group is reversible as it can be restored by anti-hypertensive therapy [11].

ACS and sudden cardiac death exhibit a prominent circadian pattern with events more frequently occurring during the morning hours (06.00-12.00 h) [12,13]. A meta-analysis suggested that approximately 1 in every 11 acute myocardial infarctions and 1 in every 15 sudden cardiac deaths are attributable to the excess morning incidence [14]. This increase begins when the subjects assume an upright posture and start the day's activities, *i.e.* during a time of sympathetic nervous system activation.

Additional epidemiological data supporting the hypothesis that sympathetic activation triggers acute coronary events come from the observation that there is an increased incidence in MI during natural disasters such as earthquakes and blizzards [13,15-17]. Interestingly, this is most evident when the disaster occurs in the morning hours, *i.e.* a combination of triggers causes the highest increase in the incidence of MI. The occurrence of ACS during sympathetic activation could result from the synchronisation of a number of potential triggers including haemodynamic alterations, vasoconstriction, and activation of prothrombotic mechanisms.

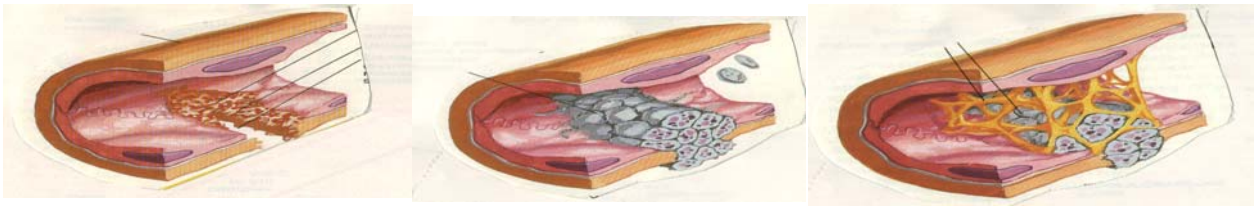
HAEMOSTASIS

Introduction

The haemostatic system contributes to a variety of body defence systems that are essential for a normal life. It impedes loss of blood and provides a system for repair of injured vascular tissue. Haemostasis consists of two interrelated stages, primary haemostasis and secondary haemostasis. The initial event that triggers a normal haemostatic response is vessel injury, which is rapidly followed by platelet adhesion and aggregation to the site of a damaged vessel, leading to the formation of a primary platelet plug in order to prevent excessive blood loss (Figure 1).

This primary platelet plug is, however, very fragile and can easily be flushed away by the blood stream and must therefore be stabilised. This stabilisation process is mediated by the second phase of haemostasis, which involves activation of the coagulation system. A series of zymogens are converted via a proteolytic cleavage into active enzymes or co-factors, which ultimately leads to the formation of thrombin, which converts fibrinogen into fibrin strands. These strands will finally cross-link to each other to form a three-dimensional network that will stabilise the platelet plug. The majority of these reactions in the coagulation system require the presence of negatively charged phospholipids exposed on the membrane of activated platelets [18]. Thus, the primary platelet plug has a very important regulatory role in that it directs blood coagulation to the site of injury.

Figure 1 Haemostasis



The steps in the haemostatic process: 1) vessel injury, 2) platelet adhesion and aggregation and 3) fibrin stabilisation of the primary platelet plug [19] (With the permission of Norvartis Sverige AB).

Very little was known about the coagulation process until about 150 years ago when Alexander Schmidt, “the father of blood coagulation”, concluded that the clotting process required two substances: fibrin generators (to day known as fibrinogen) and a fibrino-plastic substance (to day known as thrombin) [20]. In 1905 Paul Morawitz [21] reviewed the literature on blood coagulation in detail and made the first attempt to describe the coagulation system (the four factor theory). He concluded that the most likely sequence of events was that plasma prothrombin was converted into thrombin when thromboplastin (today known as tissue factor (TF)) and calcium ions were present. Fibrinogen was then converted into fibrin by formed thrombin, and unused thrombin was converted into meta-thrombin [21]. Although questions and doubts were raised, this classical theory persisted for 40 years. It was not until the late 40:s that Paul Owren examined blood from a woman with severe bleeding tendencies that the classical theory could be rejected. He found that his patient lacked an unrecognised factor, which he called factor V (as four factors previously was known). A series of additional coagulation factors were then rapidly identified by other investigators, often through studies of blood from patients with bleeding tendencies. Since different labora-tories sometimes identified the same factors without being aware of it, the factors were sometimes known under several different names. A nomen-clature committee was therefore set up in 1954, which decided that each well-characterised coagulation factor should be given a roman number, *i.e.* from FI to FXIII, which in 1954 was the number of identified factors involved in blood coagulation. Unfortunately, the roman numbers of the factors do not indicate their position or function in the coagulation system but simply the chronological order in which they were identified, FI being fibrinogen and FII pro-thrombin etc. This can sometimes be confusing as the factor that initiates the intrinsic coagulation system is actually number XII, and the very last factor, which cross-links fibrin, has the number XIII. In addition to this, FVI does not exist as it was later found to be identical to activated FV.

A decade later (1964) the coagulation process was described as an enzyme cascade for the first time by Robert MacFairlane [22] and Earl Davie [23]. This theory describes how one coagulation factor activates the pro-form of a second factor, which in turn activates a third factor and so on until enough thrombin has been formed. The name “cascade” graphically illustrates the acceleration kinetics that takes place in each of these reactions. Again for the first time, the coagulation system was divided into two systems, the intrinsic and extrinsic pathways. The intrinsic system, starting with FXII, is activated when blood comes in

contact with a foreign surface *e.g.* glass (*in vitro* activation) or a catheter, and the extrinsic system, starting with FVII, is activated when blood comes in contact with TF in the sub-endothelium. New factors that are involved in blood coagulation are still being discovered, but instead of roman numbers, they are often given names that tell us something about their function *e.g.* anti-thrombin, anti-plasmin, thrombomodulin (modulates the function of thrombin), tissue factor pathway inhibitor, thrombin activatable fibrinolysis inhibitor (TAFI) etc.

Platelets

Blood platelets are small cells that lack a nucleus, but they have a highly organised cytoskeleton, unique receptors, and specialised secretory granules. Donné discovered platelets in 1842 (globulins of chyle) [24], and 40 years later they were re-named blood platelets by Giulio Bizzozero [24]. After almost a century of oblivion they were "re-discovered" in the 1960s, and today interest in platelets and their functions is probably greater than ever judging by the 4000 publications on the subject in 2006 (PubMed).

Platelets are produced by mega-karyocytes in the bone marrow and circulate as discoid structures with a diameter ranging from 2-5 μm, and they remain in the circulation for 7 to 10 days. Young platelets are bigger and more active than old ones, and they are removed from the circulation by macrophages, mainly in the spleen, by an unknown mechanism. The amount of platelets that is required in order to maintain normal haemostasis is only 15-20% of the total number present in the circulation [25].

Platelet aggregation at sites of vascular injury is essential for the formation of the primary haemostatic plug ("a good guy") in order to stop bleeding, but also for the develop-ment of a pathological thrombus (a "bad guy") at the site of a ruptured artherosclerotic plaque. Adhesion of platelets to the sub-endothelium (the first line of defence against bleeding) is mediated via membrane glycol-protein (GP) receptors, either directly to collagen or indirectly via von Willebrand factor (vWF). The signal supplied by collagen binding will subsequently lead to platelet activation and release of new agonists, such as adenosine diphos-phate (ADP) and thromboxane A_2. In addition to this, TF-mediated thrombin generation at the surface of activated platelets will further

stimulate platelet activation and recruitment in order to secure formation of a platelet plug that is big enough to stop bleeding.

The process of platelet aggregation is the final step in primary haemostasis, which involves activation of the fibrinogen receptor on the platelet membrane. This receptor can bind to one end of the fibrinogen molecule or vWF, whereas the other end of the same molecule binds to GPIIb/IIIa on an adjacent platelet, thereby forming a three-dimensional network of "cross-linked" platelets.

Blood coagulation

After the first acute arrest of blood loss due to the formation of a loose primary platelet plug, secondary haemostasis, or coagulation, is initiated in order to stabilise the platelet plug with a fibrin network.

The proteins involved in blood coagulation, *i.e.* coagulation factors are a family of highly glycocylated proteins, and all except prothrombin and fibrinogen are found at low concentrations. With the exception of TF, which is a membrane-bound protein, they are all plasma zymogens or pro-cofactors, which require a proteolytic activation step.

Blood coagulation is a process that goes on continuously though at a very low level. A physiologically relevant activation does not occur until the endothelium is disrupted and the subendothelium is exposed to blood. Activated FVII (FVIIa), which can be generated via an autocatalytic mechanism [26], binds to TF in the subendothelium thereby forming a complex (FVIIa/TF) that can activate coagulation FX. Activated FX (FXa) alone is a rather weak activator of prothrombin but, still, enough thrombin is formed to initiate fibrin generation. This part of blood coagulation is normally referred to as the "extrinsic pathway", but the term "initiation phase of blood coagulation" is more frequently used in recent publications [27]. The endogenous inhibitor of this pathway is tissue factor pathway inhibitor.

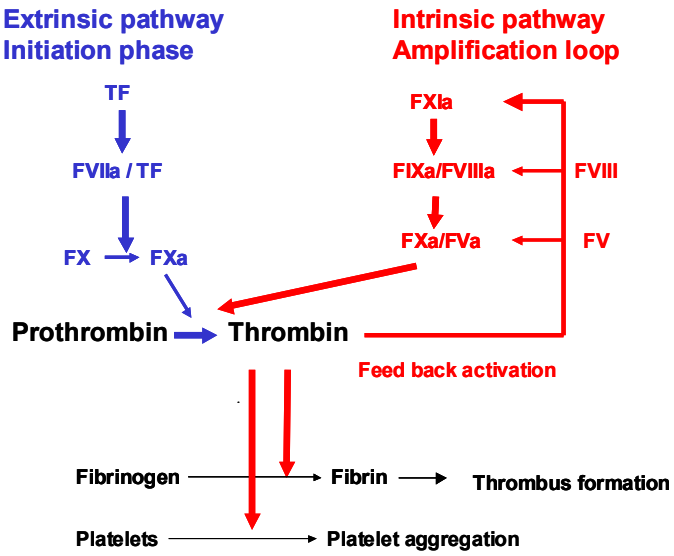
The tiny amount of thrombin that is formed during the initiation phase is crucial for the propagation of blood coagulation. Firstly, thrombin will activate and recruit more platelets to the site of injury, and secondly thrombin will activate FV and FVIII [28], which are two important co-factors in blood coagulation as they are involved in both amplification and down-regulation of thrombin generation.

Furthermore, thrombin will also activate FXI [29]. Once FXI has been activated by thrombin, it will activate FIX, which will form a complex with its co-factor FVIIIa on the surface of activated platelets. The internal tenase complex (FVIIIa/FIXa) formed is a very potent activator of FX into FXa, which is now formed at much higher concentrations compared to when FX was activated by the TF/FVIIa external tenase complex. In order to further amplify generation of thrombin, FXa forms a complex with its co-factor FVa, again on the surface of activated platelets, in order to localise thrombin generation to the site of the injury.

Finally, the prothrombinase complex (FVa/FXa) formed will activate prothrombin into thrombin at a concentration that is sufficient to generate enough fibrin for stabilisation of the primary platelet plug. This part of the coagulation cascade is known in modern literature as the amplification loop of blood coagulation [27]. It should be stressed that thrombin that is generated via this amplification loop is formed inside the growing ”haemostatic plug”, where it is bound to fibrin and prevented from inactivation by endogenous thrombin inhibitors such as antithrombin and α2-macroglobulin [27].

An addtional function of thrombin is its activation of FXIII, which, unlike the other coagulation factors, is a transglutaminase, which cross-links fibrin strands to further strengthen the “plug” and to induce its retraction [30]. Conversion of fibrinogen into fibrin and co-factor activation is not the only physiological functions of thrombin. When the concentration of thrombin within the clot reaches a certain level, thrombin starts to “leak out” from the edge of thrombus, where it will rapidly bind to thrombomodulin, which is a membrane protein on intact endothelial cells. The binding of thrombin to thrombomodulin induces a conformational change in the active site of thrombin, which results in a new substrate specificity. Instead of cleaving fibrinogen, thrombin will now cleave protein C, which in its active form (APC), and together with its co-factor protein S, can inactivate both FVIIIa and FVa. The amplification loop is thereby down-regulated, completing the entire process of thrombin generation and blood coagulation [31,32]. A schematic presentation of the coagulation system described above is given in Figure 2.

Figure 2 A schematic picture of the coagulation system



Schematic illustration of the coagulation system. FVIIa comes in contact with TF in the subendothelium, and the complex formed activates a small amount of FX to FXa, which in turn activates prothrombin to thrombin. The small amount of thrombin that is generated via this loop can then activate other coagulation factors upstream in the chain (FV, FVIII and FXI) as well as inducing activation of additional platelets. This amplification loop involves the formation of two complexes, the tenase and the prothrombinase complexes, which are very potent activators of FX and prothrombin, respectively.

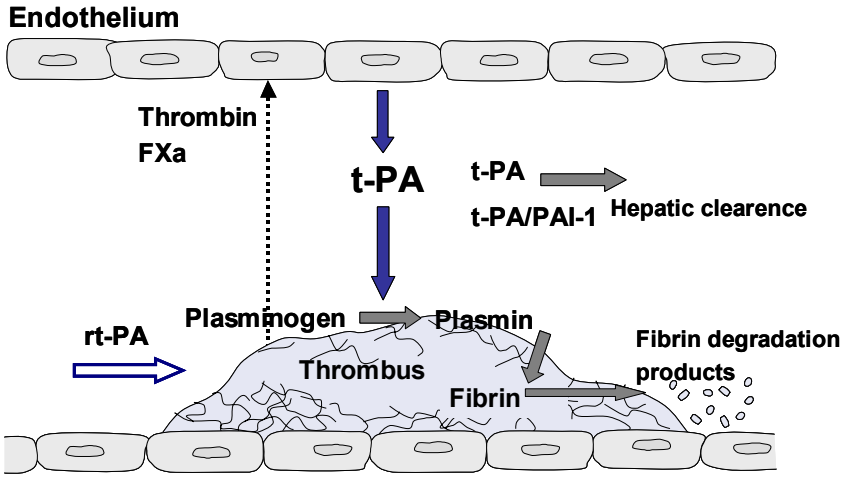
Fibrinolysis

After completion of the healing process, in which the "haemostatic plug" acts as an acute seal that will prevent excessive blood loss, the "plug" has to be removed. This process is mediated by the fibrinolytic system, which shares several similarities with the coagulation system as it involves both activation steps of pro-enzymes and co-factor functions. The central enzyme in the fibrinolytic system is plasmin, which is formed by activation of the zymogen plasminogen. The plasminogen molecule contains five lysine-binding structures, also known as "kringels", which interact specifically with certain amino acids such as lysine. As C-terminal lysine residues are exposed in partly degraded fibrin, plasminogen will bind to these sites and plasminogen will therefore be enriched on the surface of the haemostatic fibrin-rich "plug" [33].

Two plasminogen activators mediate activation of plasminogen into plasmin, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator. The latter activator is found in large amounts in urine. Its main functions are exerted in tissues, where it plays an important role in the degradation of extravascular matrix, which enables cells to migrate [34]. t-PA, on the other hand, is the main plasminogen activator in the intravascular compartment.

Like plasminogen, t-PA has also a high affinity for lysine exposed on partly degraded fibrin. Binding of t-PA to fibrin will result in a more than 100-fold enhancement of the activation rate of plasminogen to plasmin [35]. Thus, plasmin generation is a sequential and ordered activation mechanism involving the formation of a ternary complex between fibrin, plasminogen and t-PA. This mechanism will also guarantee a high local concentration of plasmin in the fibrin clot, which can now be gradually degraded into soluble fibrin degradation products of various sizes (Figure 3). Furthermore, as long as plasmin remains bound to fibrin, it has both its lysine-binding site and active site occupied, and thus can be only slowly inactivated by antiplasmin.

Figure 3 The intravascular fibrinolytic system

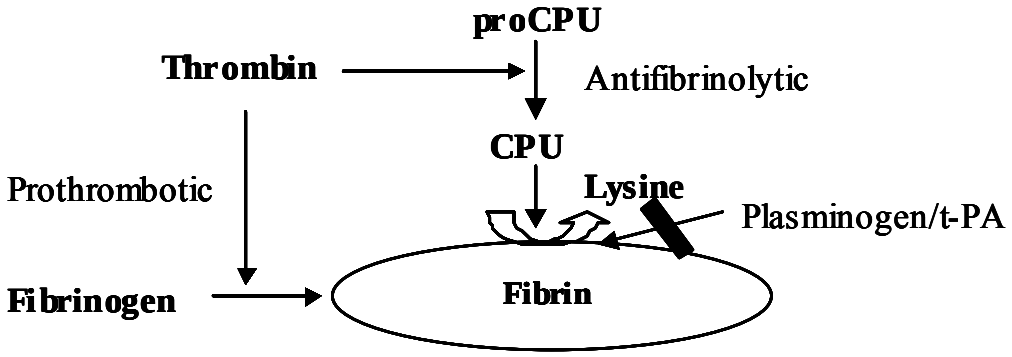


Circulating and locally released t-PA converts plasminogen into plasmin, which in turn degrades the fibrin, thereby aiding its dissolution. Substances formed during the clotting process *e.g.* thrombin and FXa are potent triggers of t-PA release. Plasminogen activator inhibitor type-1 (PAI-1) is the main inhibitor of t-PA in the vascular compartment. t-PA and t-PA/PAI-1 complex are removed from the circulation by hepatic clearance.

An important regulator of the interaction between fibrin, plasminogen and t-PA is exerted by carboxypeptidase U (CPU), also known as active Thrombin Activatable Fibrinolysis Inhibitor (TAFIa) [36,37]. The proposed mechanism of action of CPU in clotting plasma is that it removes C-terminal lysine residues from partly degraded fibrin, which results in lower plasminogen binding, and thereby lower plasmin generation and a retarded lysis rate.

Interestingly, the precursor form of CPU, proCPU or TAFI, is activated by the thrombin/thrombomodulin complex, and to a lesser extent by thrombin itself, at least *in vitro*. Thus, activation of proCPU by thrombin implies that the coagulation system plays an important role in the regulation of fibrinolysis, and that direct inhibition of thrombin will result in lower proCPU activation and thereby an increased lysis rate (Figure 4).

Figure 4 Thrombin´s role in clot formation and its stabilisation



The dual effects of thrombin; promoting thrombus formation by converting fibrinogen into fibrin (prothrombotic) and stabilising of the thrombus by converting proCPU into CPU (antifibrinolytic).

Regulation of intravascular fibrinolysis in vivo

As discussed above, t-PA is the main activator of fibrinolysis in the intravascular compartment and it is derived from endothelial cells. It is released to the circulation through a constitutive and a regulated pathway. By constitutive secretion, the newly synthesised protein continuously leaves the Golgi compartment in transport vesicles that fuse with the cell membrane. By regulated secretion, on the other hand, t-PA is released from small dense vesicles and/or the Weibel-Palade bodies [38-40]. The intracellular storage pool of t-PA is quite large, and it follows that the regulated pathway allows local intravascular t-PA concentrations to increase rapidly and substantially [41, 42]. The steady-state plasma concentration of t-PA in healthy man is approximately 5 ng/ml. It is not known whether this basal level of t-PA is maintained solely through constitutive secretion from endothelial cells or whether "steady-state stimulated" regulated secretion also contributes [43]. Plasma t-PA circulates in complex with inhibitors (mainly PAI-1) as well as in an active, uncomplexed form. The sum of complexed and uncomplexed t-PA is denoted t-PA antigen, and about 20% of steadystate plasma t-PA is in its active form. The plasma level of t-PA antigen can be determined by ELISAs based on anti-bodies that recognise all molecular forms of t-PA, and t-PA activity can be determined by functional assays, which, however, requires careful blood sampling (*e.g.* low pH) to avoid preanalytical complex binding with PAI-1 [44]. Regulated release of t-PA from endothelial cells can be induced by a number of substances with the common denominator that they activate G-protein-coupled cell surface receptors [45]. Several products formed during thrombus formation, *i.e.* thrombin and FVa as well as bradykinin and platelet-activating factor are potent inducers of t-PA release [42,43,46,47]. The same holds true for substances formed during tissue ischemia, and possibly ischemia *per se* [48-52]. Hereby, regulated release of t-PA may act as an important counter-regulatory mechanism to prevent formation of occlusive intraluminal thrombi if a clotting process is initiated. As discussed above, the clinical importance of this mechanism is illustrated by the observation that the infarct-related artery can spontaneously recanalise.

The thromboprotective role of endogenous t-PA has also been confirmed in studies in knock-out mice [53-55], which have a reduced spontaneous reperfusion after endothelial injury of the carotid artery as compared to wild-type mice. In line with this, gene therapy that induced local over-expression of t-PA, without systemic effects, prevented arterial thrombosis in an in vivo rabbit model [56].

ENDOTHELIAL CELLS

The inner surface of a blood vessel is lined with a monolayer of endothelial cells (EC) that provides an interface between circulating blood and the underlying sub-endothelium. According to the literature, the total surface area of the endothelium varies between 350 and 1000 m^2 and with a weight of 0.5-1.5 kg [57,58]. The "resting" endothelium exerts an antithrombotic effect by physically preventing blood from coming into contact with prothrombotic components such as TF and collagen in the subendothelium. The endothelium responds to mechanical, chemical, and humoral stimuli by synthesis of a wide range of biologically active mediators. Thus, it regulates vascular tone by release of vasoactive substances such as nitric oxide (NO), prostacyclin (PGI_2), and endothelin-1. Furthermore, the endothelium has a key role in haemostasis by expressing both pro- and anticoagulant substances (*e.g.* vWF and t-PA).

In addition, ECs synthesise and present on their surface molecules, which assist in this function. Anticoagulant heparan sulphate proteoglycan, which like heparin can bind antithrombin and thereby potentiate their effect, are localised to the endothelial surface [59]. Another important anti-coagulant molecule on the endothelium is TM, which is constitutively expressed in most vascular beds [60]. TM is a cell surface proteoglycan that binds thrombin, which then alters substrate specificity from fibrinogen to proCPU and protein C.

As discussed above, the fibrinolytic activator t-PA is synthesised in endothelial cells. Endothelial cells also synthesise tissue factor pathway inhibitor, which plays an important role in the down-regulation of the initial phase of coagulation [61], and protein S, which is a co-factor to APC and thereby promotes the anticoagulant activity of APC [62]. In addition to these anticoagulant and profibrinolytic effects, EC can also control the reactivity of platelets via synthesis of ecto-adenosine diphosphatase [63], prostaglandin I_2 [64] and nitric oxide (NO), also known as endothelium-derived relaxing factor [65].

The properties of EC can, however, be dramatically changed in response to various stimuli, including cytokines, endotoxin, thrombin, histamine, and hypoxia or fluid sheer stress. These changes can be due either to transcriptional responses or changes in the subcellular distribution of preformed molecules. Instead of being anticoagulant, the endothelium can in certain circumstances exhibit procoagulant and/or proinflammatory properties.

AUTONOMIC NERVOUS SYSTEM

The autonomic nervous system plays an important role in health and disease. In the healthy organism, autonomic control of a variety of organ systems serves to maintain homeostasis. Visceral and somatic afferents convey essential information to autonomic centres in the central nervous system, which in turn triggers appropriate reflex adjustments via autonomic pre- and postganglionic motor nerves. Environmental stimuli can also excite central autonomic centres. Mainly centres located in the brain stem and in hypothalamus activate the autonomic nervous system.

The peripheral auto-nomic nervous system is subdivided into the sympathetic and para-sympathetic parts, which use noradrenaline [66, 67] and acetyl-choline [68], respectively, as their main post-ganglionic chemical transmitters [69, 70]. An impaired balance between these two parts of the autonomic nervous system often occurs in association with psychosocial stress and might be involved in the pathogenesis of atherosclerotic cardiovascular diseases. It probably also helps to explain sudden cardiac death [71].

Heart rate, arterial blood pressure and a number of other visceral organ functions are closely monitored and controlled by the activity of the autonomic nervous system. Increased activation of the cardiac sympathetic fibres will increase both heart rate and cardiac contractility. Opposite effects will occur when the activity in parasympathetic fibres is increased, which results in decreased heart rate and cardiac contractility. There is a differentiation in functionality be-tween the right and left side of both sympathetic and vagal nerves to the heart. Right side nerves to the heart mainly innervate the right auricle and have a great impact on heart rate through innervations of the sinus node, whereas left side nerves mainly innervate the left ventricle and increase cardiac contractility [72].

It was only about 50 years ago that it was firmly established that the central nervous system utilises primarily chemical rather than electrical signals for communication between neurons. Much of the knowledge in neuro-sciences at that time was organised around a few basic principles, and the field had a classic simplicity.

More recently, however, there have been a growing number of reports indicating that some neurones utilise more than one chemical messenger, including a variety of peptides, and this may even be true of all neurones. The evidence that peptides could be used as neurotransmitters has greatly increased the number of potential messenger molecules, and in parti-

cular the number of co-transmitter candidates [73]. Thus, distinct patterns of synthesis and degradation of these agents, their storage and release, and receptor expression form a basis for complex co-transmission. The neuronal control of effector cells may involve both small and large molecules, (*e.g*. noradrenaline, acetylcholine, substance P, neuropeptide Y) exerting excitatory or inhibitory effects lasting for a relatively short period, in the order of milliseconds to minutes [74].

Apart from the important roles described above, the autonomic system also influences the haemostatic system. Cannon et al showed as early as 1914 that experimental animals exposed to adrenaline or pain, fear and rage had a shorter blood coagulation time [75,76]. Later, the effects of emotional excitement on blood coagulation led Selye to assume that diminished bleeding time is an integral part of the so-called alarm reaction [77]. More recently, this has been verified in man using more specific assays. Increases in the plasma concentration of fibrinogen, FVII, FVIII and vWF, as well as increased platelet activation have been observed in response to mental stress and the infusion of sympathomimetic agents [78-80]. This is, obviously, a well-adapted response as there is an increased risk of injury during a defence-alarm reaction. However, contrary to this, it was reported in the mid 1950s that anxiety might enhance fibrinolysis as measured by global methods [81-83]. Later it was shown that both mental stress and infusion of adrenaline increased the plasma concentration of t-PA, whereas PAI-1 remained unchanged [78,84].

Regulated release of t-PA *in vivo* cannot be determined by measurements of systemic plasma levels of t-PA, because it is rapidly cleared by the liver ($t_{½}$ = 3 to 5 minutes) and the plasma concentration of t-PA is therefore sensitive to any alteration in hepatic blood flow [85-88]. To overcome this problem a human perfused forearm model was developed to study local t-PA release and it was demonstrated that mental stress induces an acute release of t-PA in the forearm vascular bed, and a similar response seems to be at hand in the cerebral vascular bed [89,90]. One could thus speculate that in those vascular beds that respond with vasodilatation during activation of the sympathetic nervous system, *i.e.* the heart, brain and skeletal muscle, there is a concomitant release of t-PA. If so, this may provide a local thromboprotective mechanism against the systemic activation of procoagulant mechanisms.

However, when the present project was initiated, no investigation had yet been carried out to prove that an acute release of t-PA does occur in the coronary vascular bed in response to sympathetic activation.

ANTIPLATELET, ANTICOAGULANT AND THROMBOLYTIC AGENTS

In view of the importance of thromboembolic diseases, agents developed for the treatment and prevention of thrombosis are among the most commonly prescribed drugs in the world. Although antithrombotic agents inevitably increase the risk of haemorrhagic complications, their overall benefit far outweighs the risk. At present, the agents are classified into three groups based on their mechanism of action; *Antiplatelet drugs* – these agents inhibit platelet aggregation and are used mainly in the treatment and prevention of arterial thrombosis. *Anticoagulant drugs* – these agents act at various stages of the coagulation cascade, and are used for the treatment and prevention of deep vein thrombosis, pulmonary emboli and cardio embolic stroke but also in the acute coronary syndromes. *Thrombolytic drugs* – these agents act directly on thrombi by dissolving them, and they are mainly used for the acute treatment of myocardial infarction, but also to treat ischemic stroke and pulmonary embolism.

Antiplatelet drugs

The activation and aggregation of platelets plays a key role in thrombus formation in the heart and arterial system. Antiplatelet drugs are therefore important for the prevention and treatment of arterial thrombosis and their consequences. There are four main classes of antiplatelet drugs: 1) acetylsalicylic acid or ASA (aspirin), the most widely used antiplatelet agent; 2) $P2Y_{12}$ antagonists; 3) cAMP elevators and 4) fibrinogen receptor (GPIIb/IIIa) antagonists.

ASA remains the cornerstone of antiplatelet therapy and is routinely administered for the prevention of acute coronary syndromes and ischemic stroke. ASA is an irreversible inhibitor of cyclo-oxygenase (COX), an enzyme that is involved in the synthesis of thromboxane A_2 from aracidonic acid [91]. Thromboxane A_2 causes vaso-constriction, but it is also a platelet agonist *per se*, which can induce platelet aggregation. As ASA inhibition of COX is irreversible and platelets are incapable of synthesising new enzymes, the inhibitory effect of ASA will remain for the entire lifespan of the platelet. ASA is inexpensive and convenient, as it is available in an oral formulation, and has been widely investigated and proven to be effective. However, there are some major drawbacks of

ASA therapy, including adverse gastrointestinal events, risk of bleeding, allergic reactions and the irreversible nature of the enzyme inhibition.

$P2Y_{12}$ is a receptor on the platelet membrane that, upon activation by the agonist ADP, leads to intracellular signalling and platelet activation. In addition to preventing platelet aggregation induced by ADP, blockade of this receptor will also partly prevent aggregation initiated by other agonists such as thrombin and thromboxane A_2, as ADP is released from all activated platelets irrespective of agonist. Ticlopidine was the first $P2Y_{12}$ antagonist to be launched as an antiplatelet drug in 1980, 20 years before its target receptor was cloned and characterised [92]. The follow-up compound clopidogrel (Plavix®), a thienopyridine like ticlopidine, was approved for clinical use in 1997, and it was shown in the CAPRI study to be slightly more effective than ASA in reducing ischemic complications in patients with atherosclerotic disease [93].

A number of drugs act by increasing the concentration of cytoplasmic cAMP, which will suppress platelet activation induced by all agonists. Dipyridamole accomplishes this task via two different mechanisms. First, it inhibits the re-uptake of adenosine into red blood cells, which leads to an increased concentration of adenosine in plasma that can activate the A_{2A} receptors on platelets, which will induce cAMP production. The second mechanism of action is to prevent degradation of cAMP and cGMP by inhibiting the action of cAMP- as well as cGMP- phosphodiesterase [94].

Pharmacological inhibition of the binding of fibrinogen to its receptor GPIIb/IIIa was believed to be the ultimate antiplatelet therapy, as it would block the final common pathway of platelet aggregation independently of stimuli. Three parenteral GPIIb/IIIa antagonists are approved for clinical use in the US today: abciximab, a Fab fragment of a humanised murine monoclonal antibody; tirofiban, a synthetic molecule (non-peptide); and eptifibatide, a heptapeptide modelled from the snake venom barbourin [95]. Orally active GPIIb/IIIa antagonists have also been developed, but they all failed to show a beneficial effect in long-term clinical trials [96]. As thrombin is a very potent platelet agonist, direct inhibitors of thrombin, such as argatraoban and hirudin, will also indirectly inhibit activation of the two thrombin receptors PAR1 and PAR 4 on human platelets [97,98].

Anticoagulants

Thrombin plays a central role in thrombogenesis – its multiple actions in the coagulation cascade makes it a key target for therapeutic intervention with anticoagulant drugs. Three main classes of anticoagulant drugs are currently available: 1) heparin, including low-molecular weight heparin and a synthetic penta-saccharide, 2) vitamin K antagonists (VKAs) and 3) direct thrombin inhibitors (DTIs).

It has long been known that heparin requires a plasma co-factor for its anticoagulant action, and during the 1970s several lines of evidence indicated that this co-factor was identical to antithrombin, which is a physiological inhibitor of both thrombin and FXa [99]. Un-fractionated heparin (UFH) is currently obtained from the gastro-intestinal lining of pigs or from bovine lung and it is a heterogeneous mixture of polysaccharide chains ranging in molecular weight from 3000 to 30,000 Daltons.

More recently, low-molecular weight heparins (LMWHs) such as enoxaparin (Lovenox®/Klexane®) and dalteparin (Fragmin®) have been introduced into clinical practice [100, 101]. They are manufactured by enzymatic or chemical degradation of UFH into smaller polysaccharide chains with a molecular weight ranging from 2000 to 10000 Dalton. LMWHs are mainly used as prophylaxis against deep vein thrombosis (DVT) during haemo-dialysis and in patients undergoing surgery and as treatment of DVT and pulmonary embolism. In arterial indications it is used as thrombo-prophylaxis in patients with unstable angina and non-Q-wave infarction and treatment of ACS and in combination with thrombo-lytics. The pentasaccharide structure in heparin [102], which is responsible for binding of antithrombin, has recently been synthesised on an industrial scale and launched as fondaparinux (Arixtra®) for the same indications as LMWAs [103].

Warfarin and other VKAs exert their anticoagulant effects indirectly by inhibiting epoxy reductase. Vitamin K is a co-factor that is essential for the synthesis of γ-carboxy-glutamic acid in the liver [104]. This amino acid is present in several coagulation factors (FII, FVII, FIX and FX) and is essential for the proper function of the coagulation enzymes. The half-lives of the vitamin-K-dependent clotting factors are quite long, for example, that of prothrombin is 60–72 hours. Consequently, functional clotting factors must first be eliminated from the circulation before they are replaced with factors without γ-carboxy-glutamic acid. This means that warfarin has a delayed onset of action and, in situations where acute therapy is required; it must be administered in conjunction with

faster-acting anticoagulants such as heparin for the first few days. In addition to its anticoagulant effect, warfarin inhibits the action of the anticoagulant proteins C and S and therefore also has the potential to exert a procoagulant effect. VKAs are mostly used for stroke prophylaxis in patients with atrial fibrillation and prevention of venous thrombo-embolism. VKAs are inexpensive but, unfortunately, associated with several drawbacks such as food and drug interactions. The dose-response curve is steep and there is a need for dose adjustment to avoid bleedings cerebral haemorrhage.

As the term suggests, DTI agents exert their antithrombotic activity through direct inhibition of thrombin. Hirudin was originally isolated from the salivary glands of the medicinal leech *Hirudo medicinalis*, although it is now synthesised using recombinant technology [105]. Two recombinant forms are currently available for specific indications – lepirudin (Refludan®) and desirudin (Revasc®) [106,107]. Other DTIs include bivalirudin (Angoimax®), a synthetic version of hirudin, and argatroban, which is a synthetic derivative of the amino acid arginine [108]. Ximelagatran (Exanta®), a oral prodrug of melagatran, was shown in phase III studies in patients with atrial fibrillation to be as effective as warfarin but associated with less severe bleedings and greater comfort for the patients as there was no need for drug monitoring and dose sdjustments [109]. Unfortunately, ximelagatran, which was approved for DVT prophylaxis, had to be withdrawn from the market before it was approved for this long-term indication as it turned out to give unacceptable transaminase increases in about 6-8% of the patients (>3 times upper level of normal). However, the extensive clinical program that was performed with ximelagatran clearly demonstrated that direct thrombin inhibitors are a promising new class of oral anti-coagulants for venous as well as arterial thromboprophylaxis.

Thrombolytics

In the era before the introduction of fibrinolytic therapy for the treatment of acute myocardial infarction with ST-elevation, mortality during the first month after the event was approximately 13% [110]. Shortly after it was demonstrated that an intracoronary thrombus was the predominant underlying patho-physiological cause of transmural infarction, several pharmaceutical companies started to develop drugs that facilitated the conversion of plasminogen into plasmin. The first generation of fibrinolytic drugs comprised urokinase and strepto-

kinase. They are both effective, but not fibrin-specific. They activate circulating plasminogen into plasmin, which, in turn can degrade circulating fibrinogen as well as clot bound fibrin. Furthermore, streptokinase, which is produced from various strains of streptococci, is highly immunogenic, implying that a patient can only be treated with streptokinase once.

The second generation of fibrinolytics comprised rt-PA [111] and single-chain uro-kinase type plasminogen activator [112]. They are both recombinant proteins more or less identical to the endogenous human fibrinolytic activators, and are more fibrin-specific than streptokinase. It was therefore hoped that they would have the same efficacy as endogenous t-PA while reducing the systemic lytic state (fibrinogenolysis) and thereby the risk of bleeding. However, while a modest reduction in mortality was achieved, there was an increase in bleeding, especially haemorrhage. [113].

The third generation of fibrinolytic agents comprises *e.g.* reteplase, tenecteplase and lanote-plase, which are all designed mutants of t-PA. Reteplase consists of the kringle 2 and protease domain (K2P) of t-PA [114]. Reteplase for clinical use is produced in *E coli* and is therefore not glycocylated, which means that it has a longer half-life and can be administered as a double bolus instead of a constant infusion. Tenecteplase is a bioengineered form of t-PA that has been modified at three sites to create a molecule that has a longer half-life, higher fibrin specificity and less interaction with PAI-1 [115].

Finally, lanoteplase is derived from t-PA by deletion of finger and epidermal growth factor domains and a point mutation N117Q. Deletion of the finger-like and EGF domains results in a slower clearance. The point mutation at 117, which is a glycocylation site in wild-type t-PA, prolongs the half-life by preventing clearance by the mannose receptor [116].

The overall evidence for the benefit of fibrinolytic treatment in STEMI is overwhelming provided that the patient is treated within 12 hours of the onset of symptoms. In recent years, despite the improvement in thrombolytic drugs, PCI has become the preferred option for treatment of STEMI, provided that it can be performed within 90 minutes after the first medical contact [117]. However, this requires an experienced team, including not only interventional cardiologists but also skilled supporting staff.

ANIMAL MODELS OF THROMBOSIS

Although examples exist of naturally occurring hyperlipidaemia and atherosclerosis in a variety of animals [118], there are few examples of spontaneous thrombosis. Thus, in order to study prevention or treatment of thrombotic diseases most investigators have required the use of artificial means to introduce thrombosis in animals. Most frequently this involves some kind of mechanical or chemical damage of the blood vessel, or exposure of blood to a foreign surface in order to elicit a thrombotic response. In most cases, the experiments are acute or subacute in nature, and therefore vulnerable to the criticism that they may not properly represent the sequence of events that occurs in clinical thrombotic diseases. In addition, species differences might introduce difficulties in extrapolating the results to the human condition. Established drugs with a known mechanism of action and a well-documented effect in humans must therefore always be included as reference compounds when new antithrombotic or antiplatelet drugs are evaluated in various *in vivo* models. It is also worthy of note that the composition of thrombi varies between different models. Virchow recognised that blood flow plays an important role in thrombosis, and consequently thrombi formed on the arterial side have a different composition from those formed on the venous side. As discussed above, arterial thrombosis often follows the rupture of atherosclerotic plaque or intra-plaque haemorrhage. In this situation, high intra-stenotic shear stress may activate platelets, promoting the initial platelet-rich "white-head" of arterial thrombi, while low post-stenotic shear stress may promote the subsequent fibrin and red cell-rich "red tail". A thrombus formed on the venous side, on the other hand, is characterised by a high fibrin and erythrocytes content.

Fibrin-rich thrombus models

One common problem with all thrombosis models is to quantify the thrombus mass. A simple, but rather crude, way is to visually inspect the thrombus as in the classical Wessler stasis thrombosis model [119], which was frequently used during the early development of LMWH [120,121]. In short, the model first involves a segment of the jugular vein being isolated *in situ* before the test compound is injected intravenously. Ten to thirty minutes later, thrombus formation is initiated by an intra-

venous injection of contact-activated human plasma, and a 2 cm long segment of the jugular vein is then immediately isolated with surgical clamps. A further ten minutes later the isolated vessel is cut out and its content poured into a Petri dish with saline, where the clot can be visually examined and scored on a scale from 0 to 4, on which 0 represents no clot at all and 4 a big clot that forms a cast of the vessel. The thrombus mass in this model is formed in the absence of flow and must therefore be regarded as a clot that is formed in an "endothelialised test-tube" rather than a "true" thrombus.

Another model that better mirrors deep vein thrombosis in man, and that also entails a more precise way of determining the size of the thrombus, has been described by Hladovec [122]. This venous thrombosis model in rats fulfils all three of Virchow´s proposed criteria for thrombus formation, *i.e.* hyper-coagulability, vessel wall damage and reduced blood flow [24]. This model was, with some minor modifications, frequently used in the early development of direct thrombin inhibitors [123]. The thrombogenic factor in this model is thrombo-plastin (hypercoagulability), which is injected intra-venously about 30 minutes after the surgical procedure (vessel wall damage), during which the cava vein is exposed and all side branches tied off. Thirty seconds after injection of thromboplastin, the vena cava is tied off (stagnant blood flow) with a snare immediately below the left renal vein. The preparation is then left for ten minutes, after which the isolated segment is extirpated and the thrombotic mass dried beneath moistened filter papers before the weight is recorded. However, batch to batch variations in the activity of TF, the active ingredient in thrombo-plastin, is a large source of error in this model, and other thrombogenic stimuli, such as ferric chloride [124], platinum wire [125], intravascular foreign surface *e.g.* silk thread combined with partial stasis [126], or surgical trauma in combination with partial stasis [127], are now more frequently used.

Various metals have also been frequently used as a thrombogenic surface in bigger laboratory animals such as dogs and pigs. Already in 1940 Pearse described how metal tubes, tubular coiled springs, or flat springs could be used in dogs in order to produce thrombi in coronary arteries [128]. This coronary artery thrombosis model was later modified and used by several investigators, and the model turned out to be very useful during the early preclinical evaluation of tissue-type plasminogen activator and related thrombolytic drugs [129-131].

The thrombosis model that has been used in three of the studies in the present thesis was first described by Balir et al [129] and later slightly modified by Kordenat et al. [132]. In

short, a left fourth-intercostal space thoractomy was performed, and the heart was suspended in a pericardial cradle. A 5-8 mm segment of the LAD artery was carefully isolated, and a flow probe was placed around the LAD artery. A thrombogenic copper coil was advanced into LAD under fluoroscopy via a guide wire in the left carotid artery and placed proximal to the flow probe. The guide wire was then removed, and an occlusive thrombus was formed within 3 to 10 minutes. As thrombus formation is very rapid in this model, it is fibrin rich thereby diffracting from a typical arterial thrombus with its white, platelet-rich head. Antiplatelet drugs have therefore seldom been tested in this copper coil model.

Platelet-rich thrombus models

Electrical injury of blood vessels has been used to induce thrombosis for decades. Electrical methods of thrombus induction offer the advantage of a more precise quantification of the injurious stimulus, and thereby a more controlled degree of intimal and medial damage with exposure of subintimal structures such as collagen, elastin and TF.

Furthermore, in this model, the vessel lumen can be easily narrowed with a pneumatic occluder in order to obtain a defined disturbance in flow, which in combination with the vessel wall damage will result in thrombus formation. Time-to-thrombus formation is typically much longer, 60 minutes in this model compared to <10 minutes in the copper coil model. Thus, there is plenty of time to build up a typical arterial thrombus with a platelet-rich white head and a red tail that is rich in erythrocytes. Modifications of this technique have been frequently used to stimulate thrombus formation in rat carotid [133,134] and canine coronary arteries [135,136]. Thrombi formed in these models are, however, often of an "irreversible" nature, *i.e.* they are not prone to cause spontaneous emboli and cyclic flow changes. Such acute periodic platelet mediated thrombus formations followed by a spontaneous embolisation can, however, occur in patients with stenosed femoral or popliteal arteries. They have periodic leg pain as a result of periodic declines in leg blood flow at rest. In addition, cyclic flow reductions have been detected by measuring coronary blood flow in patients with unstable angina who are undergoing percutaneous transluminal coronary angioplasty. Thus, the symptoms in these patients are caused by platelets that interact with damaged arterial walls, periodically forming acute platelet-rich thrombi.

Folt has developed a model that mimics these events of acute platelet activation and aggregation. The principle underlying this model is that platelets passing a stenotic artery with intimal damage will adhere, first to exposed collagen and then to each other. The stenotic artery is obtained experimentally with an external adjustable plastic cylinder and external squeezing of the vessel with a surgical clamp to remove the endothelium [137-140]. Platelet aggregates build up gradually over 3 to 10 minutes and occlude the artery. As the artery becomes occluded, a pressure gradient builds up across the stenosis and may force the platelet thrombus through the stenosed lumen and into the distal circulation, thus restoring blood flow. In many cases, however, the platelet-mediated thrombus will adhere to the stenosed lumen and must therefore be dislodged mechanically by flicking the outside of the vessel with one's finger or a cotton bud.

SUMMARY OF BACKGROUND AND AIMS

As discussed, t-PA is the main fibrinolytic activator in the vascular compartment. Its release from endothelial cells may influence vessel patency following acute coronary thrombosis. Sympathetic activation increases the risk of thrombotic events, and this may in part be explained by the fact that it induces procoagulant alterations in the systemic circulation. However, it has also been shown that the systemic concentration of t-PA is enhanced not only after physical or mental stress but also after systemic infusion of different adrenergic compounds.

As t-PA has a short half-life and is cleared by the liver, these results do not give any information about whether the mechanism is increased endothelial release or reduced hepatic clearance. This is of interest as a local induction of t-PA release might oppose stress-induced procoagulant activation and thereby protect against thrombus formation during sympathetic activation.

t-PA is not only an endogenous thrombo-protective enzyme, it can also be used pharmacologically. Thrombolytic therapy with rt-PA is well documented in patients with acute MI, but the treatment is limited by a fairly slow reperfusion rate and early reocclusion in a significant number of patients. It has been demonstrated that thrombi contain a significant amount of active, fibrin-bound thrombin, which is protected from inhibition by the heparin-antithrombin complex [141].

One mechanism behind early reocclusions might therefore be that active thrombin is released into the circulation during thrombolytic therapy. Consequently, one way of improving the efficacy of rt-PA may be to combine this treatment with a low-molecular weight direct thrombin inhibitor that inhibits active fibrin-bound thrombin [142].

A new role of thrombin has recently been described in that it activates proCPU, which in its active form, CPU, hampers endogenous fibrinolysis. Thus, inhibition of thrombin may therefore also theoretically involve a pro-fibrinolytic effect in combination with its anti-coagulant effect in the coagulation cascade.

Against this background, the main **AIMS** of this thesis were as follows:

- to test the hypothesis that cardiac sympathetic nerve stimulation induces a local t-PA release in the coronary vascular bed, and if so, to investigate the mechanisms behind this response (Paper I)

- to test the hypothesis that a low-molecular weight thrombin inhibitor facilitates rt-PA-induced thrombolysis and prevents early re-occlusions (Paper II)

- to test the hypothesis that CPU is formed locally in the coronary vascular bed during pharmacological thrombolysis, and if this activation can be prevented with a low-molecular weight thrombin inhibitor (Paper III)

- to test the hypothesis that direct inhibition of CPU has a similar effect to that of a direct thrombin inhibitor regarding facilitation of rt-PA induced thrombolysis (Paper IV)

MATERIAL AND METHODS

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and approved by the ethical committee for animal research at the University of Göteborg, Sweden. The animals were all healthy and purchased from domestic breeders. They were stalled in house for at least one week before being included in the experimental protocol.

Description of the animal models – Paper I

A porcine model was used in study 1 in order to investigate whether a myocardial release of t-PA is induced during cardiac sympathetic nerve stimulation (series 1). A second porcine model was then used in order to study if the t-PA release could be explained by an increased heart rate or an enhanced local blood flow (series 2 and 3). Finally, the question whether a myocardial release of t-PA could be induced by infusion of agents acting on α- and β-adrenoreceptors was investigated (series 4). The pigs were first given an intramuscular injection of alphaxalone (Saffan®) and then about 20 minutes later a bolus dose followed by a continuous infusion of alphaxalone and α-chloralose via the ear vein. After the first set of experiments, alphaxalone was withdrawn from the market and was therefore replaced with an intra-muscular injection of midazolan (Dormicum®) and ketamine hydro-chloride (Ketaminol®) in series 2 to 4. The oesophageal temperature was moni-tored and kept within 38±0.5°C with a thermal table and heat lamp. After induction of anaesthesia, the pigs were intubated with an end tracheal cuff tube and ventilated with room air supplemented with 10% oxygen by means of a positive pressure respirator. The respiratory rate was held constant at 15 cycles per minute. Before and during the experiment, arterial blood gases and pH were adjusted to physiological levels by regulating the tidal volume and loss of fluid was compensated by a continuous infusion of Ringer® solution. Overall for series 1 to 4; a polyethylene catheter was inserted via the right saphenic artery into the abdominal aorta to record blood pressure (MAP) and collect arterial blood samples. Needle electrodes were attached to the animals for ECG lead II recordings. ECG, blood pressure and coronary blood flow were recorded and analysed by a custom-made computer program (PCLAB or PharmLab) [143]. An equilibration period of at least 30 min was allowed after the surgical preparation before the actual experiment started with baseline recording.

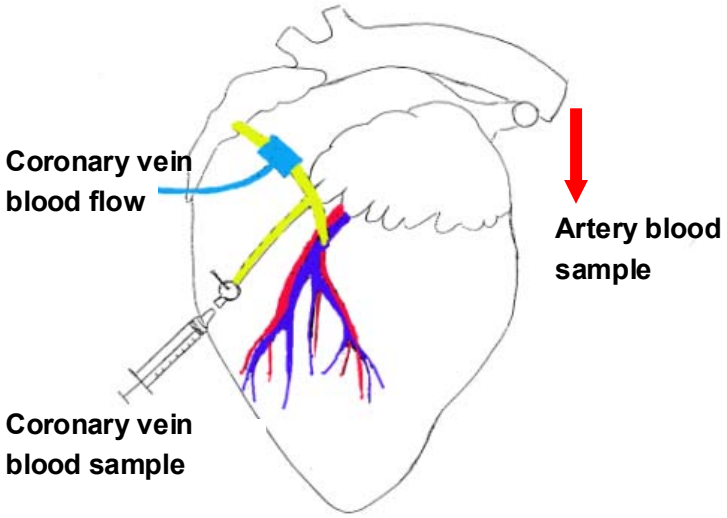
Stimulation of cardiac sympathetic nerves – Paper I series 1

The main purpose of these experiments was to test the hypothesis that cardiac sympathetic nerve stimulation induces a local t-PA release in the coronary vascular bed. An incision was made in the necks of 7 pigs, and a bilateral vagotomy was carried out. A second incision was then made straight through the thorax in the space between the clavicles and the first rib. The right and left stellate ganglions were carefully isolated from connective tissue. Each of the two ansae subclaviae was attached to silver ring electrodes and connected to a computerised constant current stimulator. After a median sterno-tomy, a shunt from a coronary vein to the right auricle of the heart was established. An in-line flow probe and a 3-way stopcock for blood sampling were placed in the shunt line. The shunt line was established in the following way: the coronary vein running in parallel to the LAD, draining the ventral part of the heart corresponding to the area supplied by LAD, was gently exposed and cannulated retrogradely with a polyethylene catheter (Figure 5). The catheter was fixed to the vein with a ligature and the other end was inserted into the right auricle. The shunt permitted rapid blood sampling from the coronary vein and facilitated exact timing of the sampling procedure relative to the start of stimulation. Immediately after the shunt was established, the pigs received a bolus dose followed by a continuous infusion of Heparin® in order to prevent clotting in the shunt.

The experiment started with 10 minutes' recording of baseline parameters, before the sympathetic nerves were stimulated with a supra-maximal current, in order to recruit all nerve fibres. The effect of mild (1 Hz) and submaximal (8 Hz) sympathetic nerve stimulation, each over a period of 3 minutes, was studied. Each animal was left to recover for at least 30 minutes between the two stimulation periods.

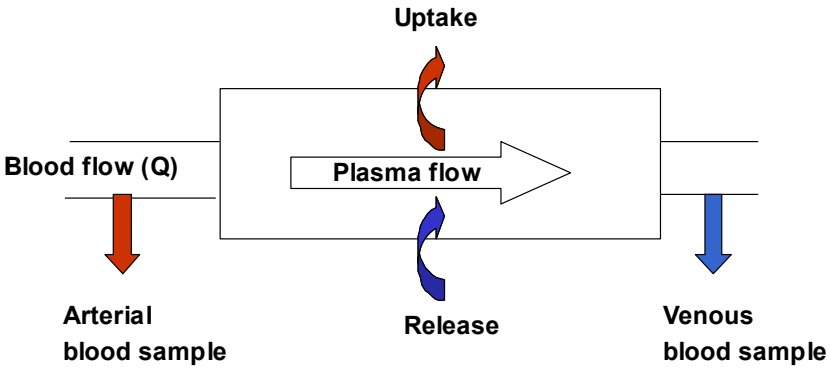
The myocardial net release or uptake was calculated in the following way; arterial (from abdominal aorta) and venous (from the coronary vein-right auricle loop) blood samples were taken simultaneously before and after 2 minutes of sympathetic nerve stimulation as well as at 1 and 5 minutes after the stimulation was stopped and 10 minutes after the end of the last 8 Hz stimulation. The first 3 to 4 mL of blood was always discarded. Blood samples were collected in syringes containing sodium citrate buffer. Plasma was isolated within 15 minutes by centrifugation, and the samples were immediately frozen and stored at -70°C before t-PA was analysed [144]. Coronary net release or uptake of t-PA was calculated according to Fick´s principle (Figure 6) [145].

Figure 5 A local vein -auricle shunt in the anaesthetised pig – Paper I series 1



Schematic drawing of the cardiac vein right auricle shunt with in-line flow probe and a 3-way stopcock for blood sampling.

Figure 6 Organ model

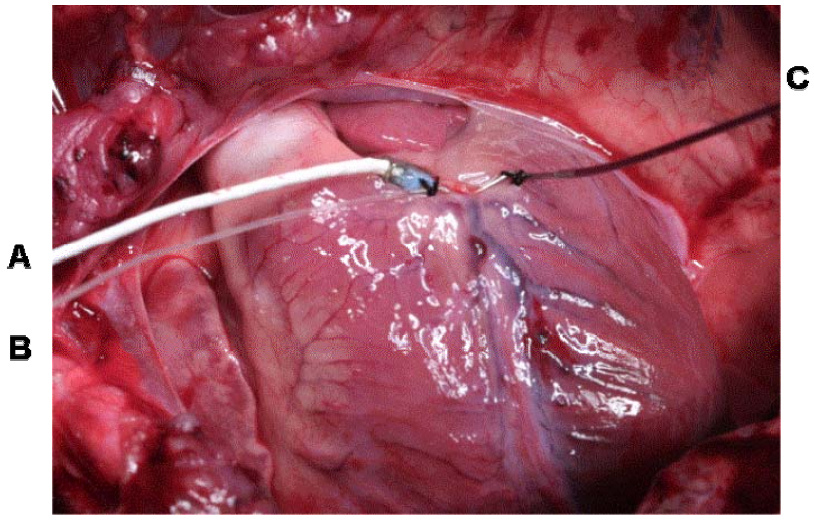


Based on the principle of Fick, net release/uptake per unit of time can be calculated from the product of the arteriovenous concentration gradient ($Cp_{venous\ plasma} - Cp_{arterial\ plasma}$) of the respective protein and local plasma flow $Q \times (100\text{-}Hct)$, Q = blood flow, Hct = haematocrit, Cp = protein concentration in arterial and venous blood respectively. Net release/uptake $= Q \times (100\text{-}Hct) \times (Cp_{venous\ plasma} - Cp_{arterial\ plasma})$.

Effects of tachycardia and hyperaemia - Paper I series 2 and 3

The main purpose of these experiments was to test the hypothesis that tacycardia and hyperaemia *per se* induces a local t-PA release in the coronary vascular bed. Seven pigs were used to test whether increased heart rate induced by right atrial pacing (150 and 200 beats/min) or increased coronary artery blood flow, as obtained by a local coronary infusion of the NO-donor sodium nitroprusside (SNP) or the ultra-short-acting Ca-antagonist clevidipine, induces t-PA release. After sternotomy, a 5-8 mm segment of the LAD was carefully isolated 50 to 80 mm from the apex, and a flow probe was placed around the LAD to measure coronary artery blood flow. By the time of this second experimental series, we had developed an alternative simplified procedure to obtain samples from the coronary vein at precisely defined time intervals. A custom-designed needle attached to a thin polyethylene catheter was used (Figure 7). This needle was placed in the local coronary vein accompanying the LAD. For intra-coronary infusions, a needle with side holes near the tip was connected to a polyethylene catheter and inserted into the LAD distal to the flow probe. For pacing of the heart, a custom-made bipolar clip electrode, connected to a custom-made computer system, was attached to the right auricle.

Figure 7 Pig model - series 2, 3 and 4



The Figure shows a pig heart with a flow probe around the LAD (A), a catheter with a needle in LAD (B), with only side holes, for intra coronary artery infusions, and a catheter with a needle in the cardiac vein (C) for drawing venous blood samples.

The experiment started with 10 minutes' recording of baseline parameters before the heart was paced for 3 minutes at 150 and 200 beats/min, respectively. After a 30-minute recovery period and a new 10-minute baseline recording, SNP was infused into the coronary artery in two dose steps: 0.1 and 0.5 mg/min for 3 minutes each. However, the infusion of SNP did not give the same increases in coronary artery blood flow as seen in series 1 without inducing other general haemodynamic effects, *e.g.* decrease in blood pressure. To avoid the systemic haemodynamic effects caused by SNP infusion, an additional series of experiments in 7 pigs was performed in which clevidipine, a calcium-antagonist with a half-life shorter than 30 seconds in pigs and dogs [146, 147], was used in order to avoid changes in blood pressure. This approach enabled us to reach the stipulated blood flow levels.

Effects of local myocardial α- and β-adrenergic stimulation - Paper I series 4

The main purpose of these experiments was to test the hypothesis that α- and β- adrenergic stimulation *per se* induces a local t-PA release in the coronary vascular Using the same preparation as above, 8 pigs were studied to evaluate whether changes in local myocardial t-PA release could be induced by infusion of α-(phenylephrine (PE)) and β- (isoprenaline (IPR)) sympathomimetic agents. PE and IPR were therefore infused in the coronary artery at increasing doses of 1, 4, 16 and 64 μg/min and 0.1, 0.4, 1.6, and 6.4 μg/min, respectively. Each dose was infused for 5 minutes in sequence. A recovery period of 1 hour was allowed between the two drugs. Because IPR was expected to induce systemic effects, the two agonists were not infused in randomised order.

Description of the animal model - Papers II, III and IV

A dog model of coronary artery thrombosis induced with a copper coil inserted into LAD was used in Papers II, III and IV.

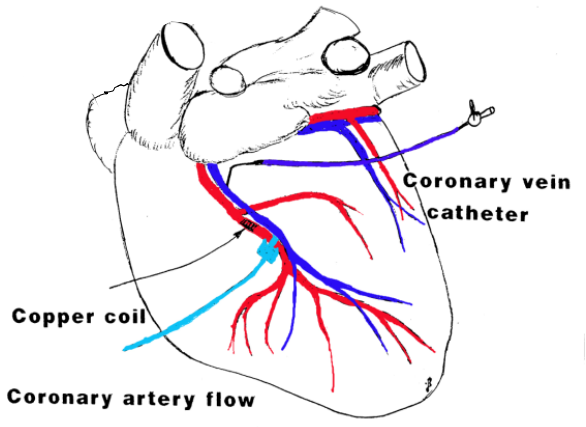
The dogs were anaesthetised with an intravenous infusion of sodium methohexital (Brietal®) followed by α-chloralose. A stable plane of anaesthesia was maintained throughout the experiment by a continuous infusion of α-chloralose. The oesophageal temperature was monitored and kept within 37±0.5°C with a thermal table and heat lamp. After induction of anaesthesia, the dogs were intubated with an end tracheal cuff tube and ventilated with room air supplemented with 10% oxygen by means of a positive-pressure respirator. The respiratory rate was held constant at 15 cycles per min. Before

and during the experiment, arterial blood gases and pH were adjusted to physiological levels by regulating the tidal volume and by infusing sodium bicarbonate. Ringer® solution was infused at a constant rate to replace fluid loss.

Cannulas were inserted into the saphenic vein for infusion of drugs, and into the radial vein for infusion of anaesthetics, sodium bicarbonate and Ringer® solution. Systemic arterial pressure was monitored with a catheter inserted into the aorta via the saphenic artery. Haemodynamic variables including coronary blood flow as well as ECG (lead II needle electrodes) were recorded with a Grass polygraph equipped with a custom-made computer program (PCLAB or PharmLab). The chest was opened by an incision in the fourth left intercostal space, and the heart was suspended in a pericardial cradle. A 5-8 mm segment of LAD was carefully isolated, and a one-mm ultrasonic transit-time flow probe was placed around the LAD artery. A custom-made thrombogenic copper coil was advanced into LAD under fluoroscopy, via a guide wire in the left carotid artery, and placed proximal to the flow probe. The guide wire was then removed and zero blood flow verified the presence of an occlusive thrombus. The thrombus was then allowed to stabilise for 30 minutes before rt-PA was infused over a period of 20 minutes.

A schematic illustration of the instrumentation in this model is shown in Figure 8.

Figure 8 Dog preparations - Papers II, III and IV



Copper coil preparation in the anaesthetised open chest dog. A copper coil 6 mm long and 1.2 mm in diameter was inserted into the LAD under fluoroscopy by means of a guide- wire system inserted via the right carotid artery. The coronary vein catheter was only used in Papers III and IV.

Drugs and experimental protocol - Paper II

The main purpose of these experiments was to see if co-administration of anticoagulants with rt-PA could improve LAD patency compared to rt-PA alone. These anti-coagulants were heparin, hirudin and melagatran.

After instrumentation, the dogs were randomised into 6 groups with 6 dogs in each group. The dog's in-group 1 served as a reference group and received a 20-minute infusion of rt-PA (Actilyse®) at a dose of 1 mg/kg. Adjunctive drug therapy to rt-PA was initiated in the other 5 treatment groups with a bolus dose followed by a continuous infusion over 90 minutes. The dogs were then observed with respect to artery patency status for another 90 minutes, and reperfusion was defined as return of LAD flow of >20% of the baseline flow before thrombus formation. Heparin has been administered in several clinical indications at doses aiming to double the activated partial thromboplastin time (APTT) [148], and it was therefore considered to be relevant to compare the compounds at this level of anticoagulation. Melagatran and hirudin were also tested at two additional dose levels in order to compare theire effect an equiemolar plasma concentration. The major endpoints in the study were 1) time to reperfusion; 2) number of reocclusions; and 3) time to first reocclusion.

Drugs and experimental protocol – Paper III

The main purpose of these experiments was to follow changes in CPU activity in coronary blood vessels before; during and after successful rt-PA induced lysis, and to investigate whether these changes were influenced by co-administration with a direct thrombin inhibitor. The experimental protocol in this study was similar to that in Paper II. Dog's in-group 1 (n=10) received a 20-minute infusion of rt-PA (total 1 mg/kg), and dog's in-group 2 (n=10) received rt-PA as in group 1 in combination with a 180-minute infusion of melagatran (0.15 mg/kg ×h).

In addition to this, a third group (n=6) of sham-operated dogs, *i.e*, without copper coils, received the same rt-PA treatment as in groups 1 and 2. Vessel patency was monitored continuously over a period of 180 minutes, reperfusion was defined as a return of coronary artery flow to a level of >0.002 L/min, and a subsequent reocclusion was docu-mented when the coronary blood flow declined below 0.002 L/min for a period >2 minutes. Study variables were similar to those in Paper II, *i.e.* time to reperfusion and mean coronary artery (LAD) blood flow during patency.

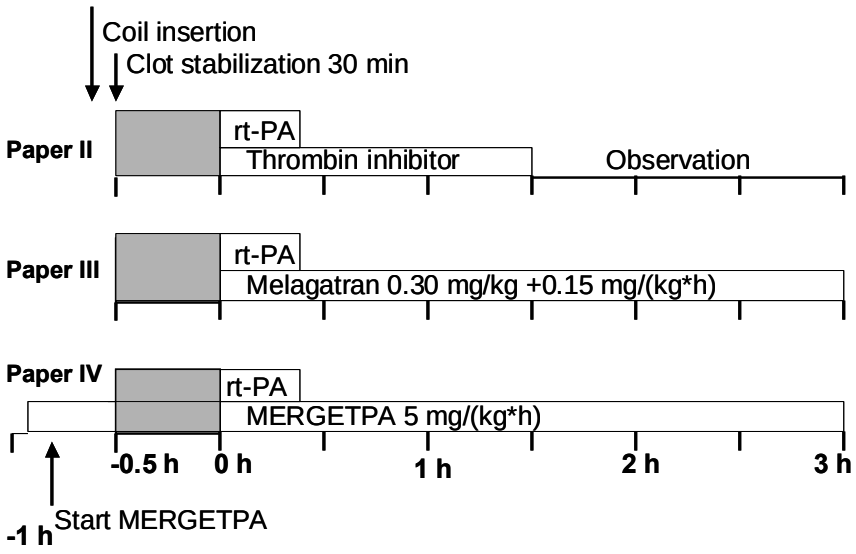
Blood for determination of CPU activity in plasma from venous and arterial blood was collected from the great cardiac vein and aorta, respectively. Blood samples were collected at baseline, immediately before and after coil insertion, before and after the start of rt-PA infusion, and then at 5–15-minute intervals throughout the period of patency. Blood was collected in test tubes containing a cocktail of sodium citrate and PPACK and aprotinin in order to avoid *ex vivo* activation of proCPU, and was then stored at -70°C until CPU analysis [149]. The amount of CPU that was generated in the coronary vessels drained by the great cardiac vein during the period of patency was then calculated according to Fick´s principle (figure 6). The total amount of generated CPU was then expressed as the area under the CPU activity versus time curve.

Drugs and experimental protocol – Paper IV

The main purpose of these experiments was to study the hypothesis that direct inhibition of CPU will lead to the same results as inhibition of thrombin-mediated proCPU activation with a thrombin inhibitor, which was discussed in Paper III.

The commercially available compound DL-2- mercaptomethyl-3-guanidino ethylthio-propanoic acid (MERGETPA) is an inhibitor of both CPU and carboxypeptidase N (CPN) activity. It has earlier been demonstrated that MERGETPA exerts a marked pro-fibrinolytic effect *in vivo* in a rat model with endotoxin-induced disseminated intravascular coagulation [150]. CPN is constitutively secreted into the circulation and plays an important role in processes not associated with fibrinolysis, *e.g.* inactivation of active peptide hormones such as kinins and anaphylatoxins. It was therefore considered to be relevant to use the anti- CPU effect of MERGETPA in order to study the effect of CPU. The experimental protocol was identical to that in Paper II with the exception that MERGETPA was first given as a bolus dose of 5 mg/kg, immediately followed by an infusion of 5 mg/(kg×h), which was started as soon as the basal haemodynamic measure-ments had been recorded, *i.e.* 30 minutes before insertion of the copper coil. This regimen with the inhibitor present in the circulation during thrombus formation aimed to demonstrate a possible prophylactic effect of CPU inhibition rather than an acute treatment effect. The experimental protocols and drug infusions used in Papers II, III and IV are illustrated schematically in Figure 9.

Figure 9 Experimental schedule - Papers II to IV



Schematic illustration of the experimental design and drug infusions in the three dog studies.

Statistics

Standard statistical methods were used. All results are given as mean values±standard error of the mean (SEM). Statistical tests were considered significant at $p<0.05$ (in Figures *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$). For further details see Paper I to IV respectively.

RESULTS AND DISCUSSION

The majority of acute coronary syndromes are caused by activation of intravascular clotting. When unopposed, this intravascular clotting can rapidly progress into formation of an occluding arterial thrombus. A common theme in this thesis is the capacity of the endogenous release, and exogenous supply of the fibrinolytic enzyme t-PA. When intravascular clotting is initiated, the vascular endothelium can respond by a rapid release of t-PA [42]. As the efficacy of t-PA is about two orders of magnitude greater when present during thrombus formation rather than after, it represents a powerful thromboprotective mechanism, which probably explains the substantial rate of spontaneous reperfusion in myocardial infarction. The thrombolytic capacity of t-PA is also used in the clinical setting, and intravenous infusion of rt-PA is a well-established treatment for myocardial infarction. A model for studying local endogenous t-PA release in the coronary vascular bed in response to sympathetic activation in the pig is described in Paper I in this thesis. A similar approach was used in Paper III, *i.e.* to investigate if there is a local release of enzymes in the dog coronary vascular bed, but then the aim was to study if pharmacological treatment with rt-PA would result in a local activation of proCPU. Finally, Papers II and IV both deal with exogenously administered rt-PA and various approaches to improving its efficacy.

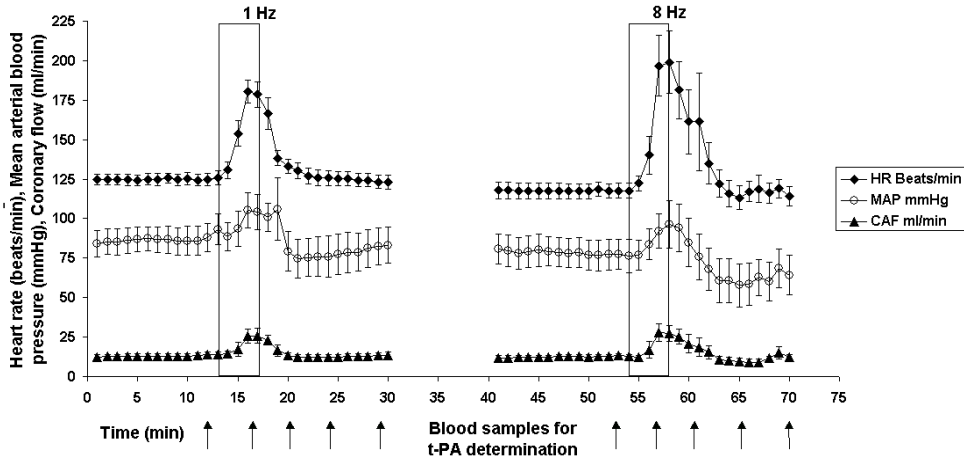
Coronary t-PA release in response to stimulation of the cardiac sympathetic nerve – Paper I series 1.

Circulating t-PA is removed from plasma by the liver with a half-life of about 3-5 min [88], and it is therefore more or less impossible to determine whether an increased systemic concentration is due to increased endothelial secretion or reduced hepatic clearance of the protein. This is especially true during sympathetic activation, which induces a reduction in hepatic blood flow and thereby the hepatic clearance of t-PA [85]. Our group therefore adapted experimental designs, both in man and in experimental animals, to be able to determine t-PA secretion [89,144]. To avoid interference from rapid hepatic clearance, these models are based on recording the plasma concentration of t-PA in venous and arterial blood that drains and supplies a local vascular bed. The net release of the protein can then be calculated as the difference in veno-arterial plasma concentration multiplied by the local plasma flow (Figure 6). This technique has been used by several investigators in order to study if there is an acute release of t-PA in response to local infusion of noradrenaline, methacholine, desmopressin, brady-kinin and substance P, and t-PA-release has been demon-

strated in the human forearm and coronary vascular bed [89,151-154]. With regard to sympathetic activation, our group has demonstrated an induction of t-PA release across the human forearm in response to mental stress [89] and across the coronary and cerebral vascular bed in response to surgical stress [90,155]. However, these mild stress factors only induce a 2-fold increase in t-PA release, which is a very weak response compared to more than 20-fold increase that has been observed in response to pharmacological stimulation. A pig model, described in Paper I, was therefore developed to investigate the capacity for an endogenous t-PA release in the coronary bed in response to sympathetic nerve stimulation as well as the physiological mechanisms behind this response.

Electrical stimulation of cardiac sympathetic nerves with a frequency of 1 and 8 Hz caused an instant and frequency-dependent increase in heart rate, mean artery blood pressure and coronary artery blood flow, as illustrated in Figure 10.

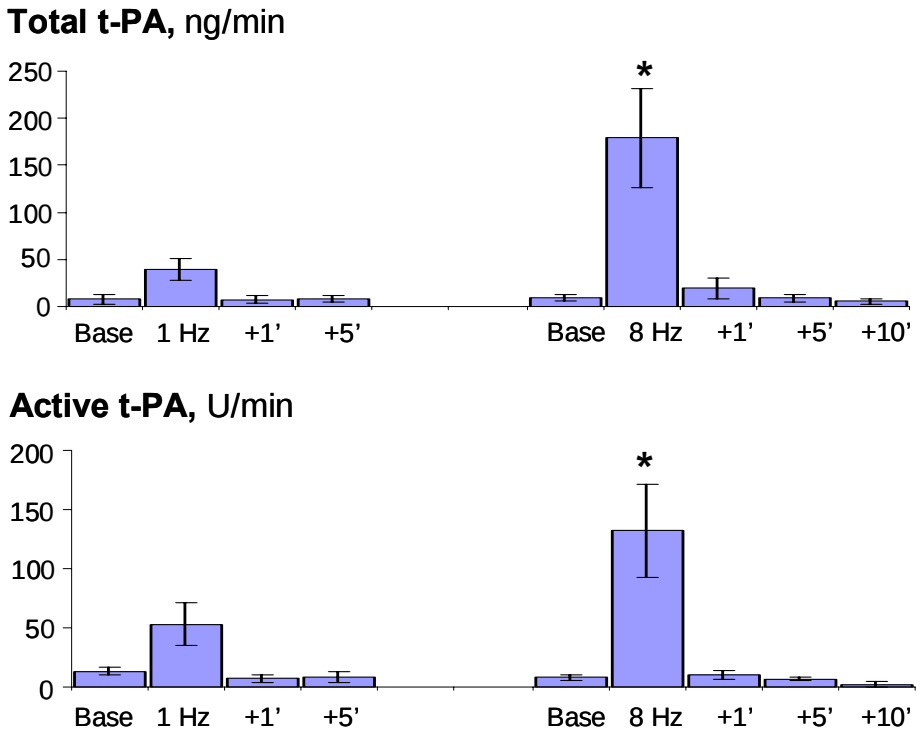
Figure 10 Haemodynamic responses to sympathetic nerve stimulation – Paper I series 1



Sympathetic nerve stimulation with a frequency of 1 and 8 Hz induced an increase in the heart rate from 125 beats/min to 180 and 200, respectively, while the mean artery blood pressure increased 15 and 20 mmHg and the coronary artery blood flow increased approximately 2- and 2.5 fold.

The baseline release of total and active t-PA across the coronary vascular bed was 7.6±5.7 ng/min and 13.4±2.9 U/min, respectively, before the first periods of sympathetic nerve activation, and 9.5±3.8 ng/min and 8.0±2.2 U/min before the second period (Figure 11). Low-frequency (1 Hz) sympathetic nerve stimulation resulted in an approximately 5-fold increase for both total and active t-PA release, whereas high-frequency (8 Hz) stimulation gave a 20-fold increase for both total and active t-PA ($p<0.05$). After nerve stimulation ceased, the net release rates of t-PA declined rapidly and returned to pre-stimulation levels within 1 minute (Figure 10).

Figure 11 Coronary net release of t-PA – Paper I series 1



Before both periods of sympathetic nerve activation there was a net baseline release of both total and active t-PA across the coronary vascular bed. A marked increase in the net release rate of both total and active t-PA was observed in response to both stimulation frequencies, but this increase was only statistically significant in response to high-frequency stimulation (statistic, compared to base values).

Effects of tachycardia and hyperaemia on coronary t-PA release- Paper I series 2 and 3

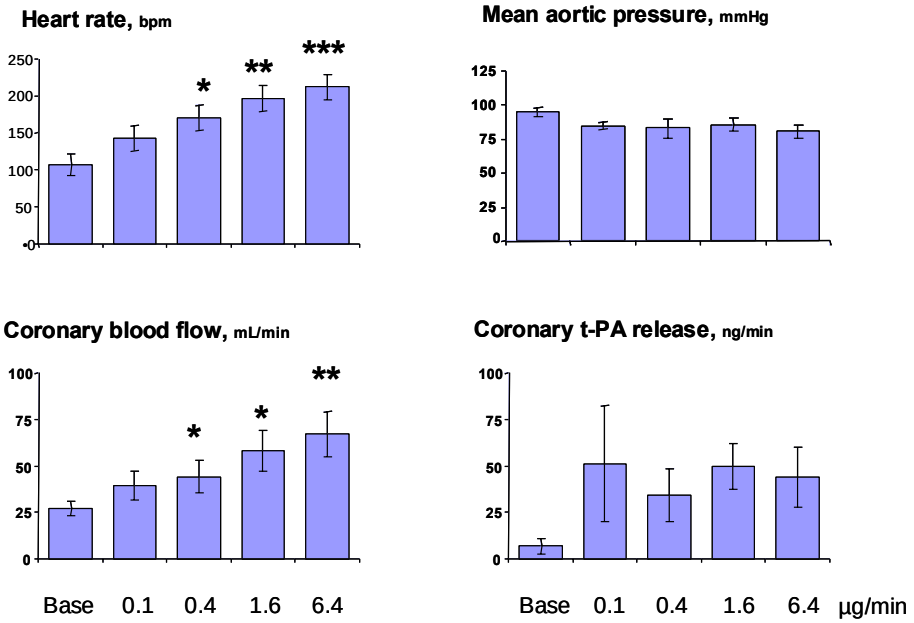
An increase in heart rate, from 125 to 150 and 200 beats/min, as induced by right atrial pacing had no influence on myocardial t-PA release. In order to investigate if coronary t-PA release is flow-dependent, the effect of a local infusion of the NO donor SNP was studied. However, due to a significant dose-dependent drop in mean artial blood pressure, it was not possible to increase coronary blood flow more than 1.5-fold, compared to the 2-fold increase that was observed in response to high-frequency sympathetic stimulation. Another series of experiments was therefore designed in which an ultra-short-acting calcium antagonist (clevidipine) was infused intra coronarily. With this approach the coronary blood flow increased dose-dependently without inducing any significant systemic effects. The mean increase in coronary blood flow at the highest dose was 2.5 times over baseline, which is in the same order of magnitude as the increase that was observed in response to high-frequency sympathetic stimulation. However, despite this, clevidipine did not induce any significant change in coronary t-PA release. Thus, the t-PA response is independent of changes in heart rate and in local blood flow *per se*. With regard to blood flow, this is in line with earlier studies showing that SNP has no effect on t-PA release in either the human forearm or in the human coronary vascular bed [8,151,156-158].

Effect of local myocardial α- and β- adrenergic stimulation – Paper I series 4

Isoprenaline induced a dose-dependent increase in both heart rate and coronary blood flow (Figure 12) as well as a significant increase in the venous plasma concentrations of t-PA. There was also an increase in the coronary net release of t-PA, which approached statistical significance ($p=0.06$). In addition, the arterial plasma concentration of t-PA increased, which indicates that IPR also had a systemic effect. The mean maximal increase in coronary t-PA net release in response to IPR was 6-fold, compared to 5- and 20-fold in response to low- and high-frequency sympathetic stimulation, respectively (Figure 12). In contrast to IPR, phenylephrine had no significant effects on either haemodynamic variables or coronary t-PA release. One problem with an intra-coronary infusion of adrenoreceptor stimulating substance is that it can induce an inhomogeneous stimulation of the cardiac muscle, and possibly causing irregular heart beats. This could be one explanation why there was no dose-dependent increase in t-PA release with higher IPR doses. The observed response (in t-PA levels) to β-adrenergic receptor stimulation is in

line with previous measurements of systemic plasma levels of t-PA [80], data obtained by computer simulation to model t-PA secretion [85] and results from isolated perfused vascular systems [159,160]. It has also been shown that IPR is a stimulus for t-PA release in the human forearm [158], and the present finding in the coronary vascular bed has been confirmed [161].

Figure 12 Haemodynamic and t-PA response to isoprenaline – Paper I series 4



Heart rate, mean aortic blood pressure, coronary artery blood flow and coronary net release of t-PA before and during increasing doses of isoprenaline infused into LAD (statistics, compared to base values).

The results of the different experimental series in Paper I are summarised in Table 1. The main and original finding in this study was that stimulation of cardiac sympathetic nerves could induce an acute release of t-PA, mostly in its free, active form. The results also show that neither haemodynamic changes nor α-adrenergic stimulation could explain the profound release of t-PA observed in response to sympathetic stimulation. In contrast, β-adrenergic stimulation may contribute. Hence, one plausible explanation is that activation of cardiac sympathetic nerves increases local norepinephrine spillover, which in turn induces a

regulated release of t-PA from endothelial stores. However, given the magnitude of the response, it is likely that the induction of the endothelial t-PA release should be attributed to other causes. One possibility is that t-PA released from the sympathetic terminals system itself contributes to the observed response. O'Rourke and co-workers have shown that sympathetic neurons can synthesise and release t-PA [162, 163]. However, whether t-PA derived from sympathetic neurons actually reaches the blood stream remains to be determined.

As mentioned in the Introduction, prothrombotic mechanisms are activated during sympathetic activation. The observed local t-PA response may thus constitute an important counterregulatory mechanism to prevent formation of occlusive intraluminal thrombi. Another porcine study has shown that coronary release of t-PA is also markedly increased after brief periods of local myocardial ischemia [52]. However, it was recently demonstrated that this response declines following successive periods of ischemia [52]. It would therefore be of interest to evaluate the response to repeat periods of sympathetic activation to fully evaluate the capacity of this response. It would also be of interest to be able to determine t-PA release in different segments of the coronary vascular bed. However, no such experimental models have been developed so far.

Table 1 **Compilation of the main findings - Paper I**

Intervention	Heart rate	Blood pressure	Coronary blood flow	t-PA release
Sympathetic stimulation	↑↑↑	↑↑↑	↑↑	↑↑↑
Increased heart rate by cardiac pacing	↑↑↑	↓	-	-
Vasodilatation Nitroprusside (NO)	↑	↓↓	↑	-
Vasodilatation Clevidipine (Ca^{2+})	-	-	↑↑	-
β-adrenergic stim Isoprenaline	↑↑↑	-	↑↑	↑
α-adrenergic stim Phenylepherine	-	-	-	-

Sympathetic cardiac nerve stimulation increases cardiac t-PA release to a higher degree than other investigated interventions.

Thrombolytic studies with rt-PA alone or combined with inhibitors of thrombin or CPU - Papers II, III and IV

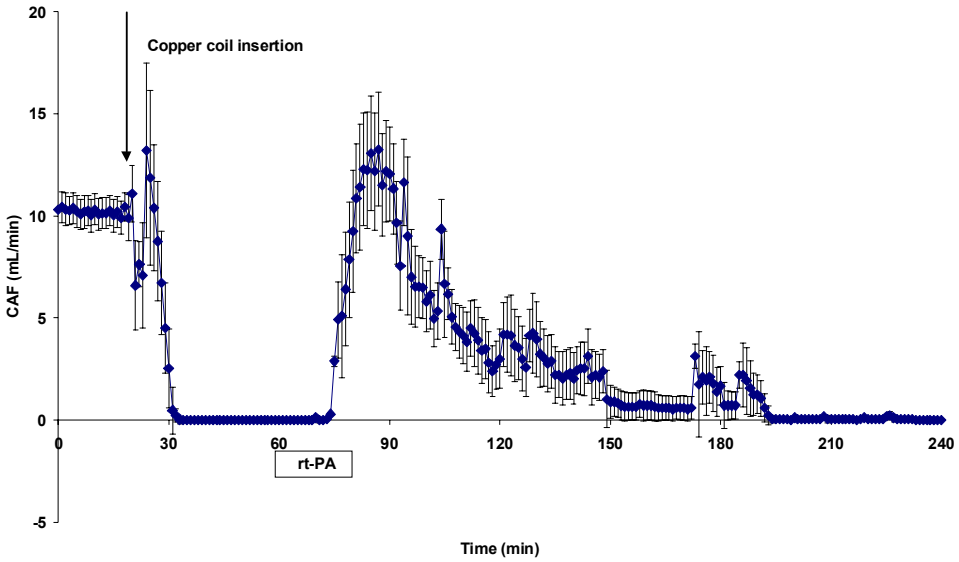
Study parameters in the rt-PA reference groups.

While release of endogenous t-PA from the coronary vascular bed was studied in Paper I, Papers II, III and IV all deal with the thrombolytic effect of exogenous rt-PA, administered as a constant infusion over 20 minutes at a dose of 1 mg/kg. One group of 6 to 10 dogs received this treatment in all studies, and this group served as a reference group for other groups of dogs that, in addition to rt-PA, received concomitant treatment with thrombin- or CPU inhibitors. The copper coils described in Papers III and IV had a more distal location in LAD than those in Paper II, so that the basal blood flow at the site of the flow probe was lower in the group of dogs concerned (10 to 12 mL/min) compared to the basal flow in study II (18 to 24 mL/min). The return of blood flow in a totally occluded vessel as a result of rt-PA treatment is sometimes characterized by a gradual increase in blood flow. In some animals, however, it may progress from "zero flow" to "maximal flow" in one or two heartbeats, probably due to sudden embolisation. The parameter "time to lysis" must therefore be carefully defined in each study. In Paper II, recanalisation was defined as the time it took to reach a LAD blood flow of 3.5 to 4.5 mL/min (20% of the basal flow in each individual dog) and time to lysis in Papers III and IV was set to a flow rate >2 mL/min. Although there were almost 10 years between study II and study IV, the basal results in the control groups from the three studies were very consistent, as seen in Table 2. The longer lysis time in Paper II with rt-PA alone is, as discussed above, explained by the fact that the coil in this study was placed in a segment of LAD with a wider diameter, which resulted in a larger thrombus. An example of the coronary artery blood flow in dogs with a copper coil inserted in LAD (rt-PA group – Paper IV) and treated with rt-PA is illustrated in Figure 13.

Table 2 Basal study parameters in the control group - Papers II, III and IV.

	Paper II	Paper III	Paper IV
Basal LAD flow (mL/min)	18-24	10-15	10-15
Limit/reperfusion flow (mL/min)	4	2	2
Time to lysis (min)	34	21	20
Patency time (first period)	18	26	18
Frequency of reocclusions	6/6	10/10	10/10

Figure 13 Coronary artery flow - Paper IV



LAD blood flow (10 dogs) response to copper coil insertion and 20 minutes of rt-PA (1 mg/kg) treatment (rt-PA group in Paper IV).

Thrombolysis and thrombin inhibition – Paper II

The objective of the study described in Paper II was to characterise the modulation of rt-PA- induced thrombolysis with three anticoagulant drugs with different modes of actions. This study was performed in the mid 1990s when adjunctive therapies with a combination of rt-PA and heparin or the direct slow-tigth binding thrombin hirudin were a very "hot" clinical issue. The Gusto IIB trial [164] showed that hirudin and heparin in combination with rt-PA produced similar effects on the combined endpoints death and myocardial infarction in patients with acute myocardial infarction. It had been suggested that thrombin trapped in, or bound to, the fibrin in a thrombus may play an important role in resistance to thrombolysis and subsequent reocclusion [37,141]. It was therefore hypothesised that a small, potent thrombin inhibitor (MW 429) could be more effective in inhibiting fibrin-bound thrombin than the heparin: antithrombin complex (MW ≈75000) and the polypeptide hirudin (MW ≈7000). Basal APTT in untreated dogs was 16.1±4 seconds, and in some preliminary dose-guiding studies the dose that doubled this clotting-time was determined to be 0.3 mg/(kg×h), 1.2 mg/(kg×h) and 50 IU/(kg×h), for melagatran, hirudin and heparin, respectively. Melagatran and hirudin were also tested at an additional dose-level in order to compare their effects at equivalent molar plasma concentrations.

Reperfusion exceeding 3 minutes in duration was obtained in all dogs, and the average time to lysis in the control group was 34±8 minutes. All compounds shortened the time to lysis when they were administered at a dose that doubled APTT (heparin 22±7 and hirudin 23±5 minutes), but it was only with the low-molecular weight thrombin inhibitor, melagatran, that the reduction of time to lysis was significant compared to that in the reference group (13±5 minutes). In order to achieve the same reduction with hirudin (12±5 minutes), the dose had to be increased to 3.6-mg/(kg×h), a dose that, however, caused unclottable blood *ex vivo* (APTT >600 seconds) and ventricular fibrillation in all dogs, and two of those died during the experiment.

All dogs in the reference group reoccluded about 20 minutes after successful lysis. This was, of course, expected as rt-PA has a very short half-life *in vivo*, and the thrombogenic copper coil was still in LAD. Anticoagulant treatment was, however, able to prevent an early reocclusion, and the time to the first reocclusion was significantly prolonged with increasing doses for both melagatran (106±7 and 162±13 minutes) and hirudin (96±16 and 170±10 minutes), whereas heparin only prolonged the patency time by about 50 minutes. However, parameters such as "time to lysis" and "time to reocclusion" are *per se* not indicators of successful treatment. The vessel can be open, but vessel constriction or a non-occlusive thrombus can counteract a normal blood flow. The blood flow (mL/min) was therefore recorded frequently during the period of patency, and the total amount of blood (mL) passing through LAD was obtained by multiplying the average flow by the patency time. The mean flow in each group is summarised in Table 3.

Table 3 Total coronary artery blood flow during patency - Paper II

Treatment	Total blood flow	Compared to	Compared to
	mL	rt-PA	Melagatran 0.3 mg/kg
rt-PA alone	452±458		
+Melagatran 0.1 mg/(kg ×h)	1348±243	p<0.05	
+Melagatran 0.3 mg/(kg ×h)	2766±154	p<0.01	
+Hirudin 1.2 mg/(kg ×h)	1322±975	p<0.05	p<0.05
+Hirudin 3.6 mg/(kg ×h)	2220±365	p<0.01	ns
+Heparin 50 U/(kg ×h)	1483±746	p<0.05	p<0.01

Differences in coronary blood flow between treatments groups were analysed with a non-parametric Wilcoxon-Mann-Whitney test as a non-Gaussian distribution was found in most of the studied parameters. This test revealed that all groups caused a significant increase in coronary blood flow compared to the reference group with rt-PA alone. A comparison between groups that gave the same prolongation of APTT also revealed that melagatran at a dose of 0.3 mg/(kg×h) gave a significantly better flow than hirudin at a dose of 1.2 mg/(kg×h) or heparin at a dose of 50 IU/(kg×h), which supports the hypothesis that a low-molecular weight direct thrombin inhibitor is more effective in inhibiting clot-associated thrombin. However, during the preparation of Paper II, some new data were presented that suggested an additional mechanism that might explain why there seems to be a pro-fibrinolytic effect of small direct thrombin inhibitors [165].

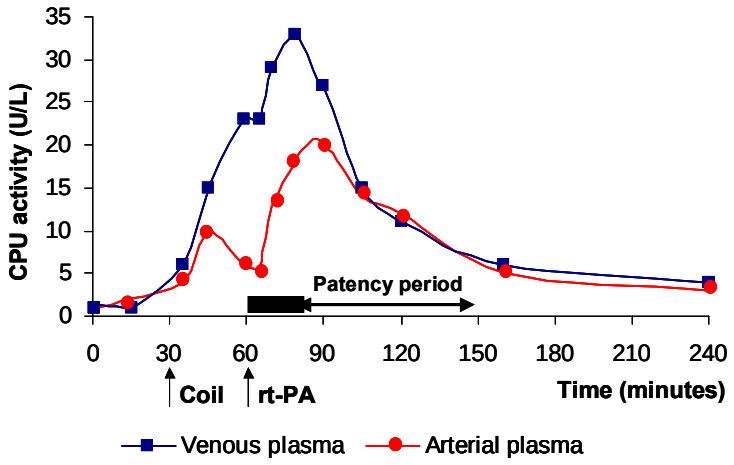
A new physiological role for thrombin was described in that it can activate the zymogen proCPU to CPU, which attenuates fibrinolysis by catalysing the removal of C-terminal lysine residues from partly degraded fibrin, thereby reducing the number of binding sites for plasminogen and t-PA on the clot surface. Thus, inhibition of thrombin in the clot will prevent proCPU activation and thereby facilitate fibrinolysis. This new insight, that thrombin also has a regulatory role in the fibrinolytic system, encouraged us to continue with our experiments, but now with more focus on CPU.

Thrombolysis and carboxypeptidase U - Paper III

The CPU activity in coronary venous and arterial plasma was analysed before copper coil insertion, during thrombus formation, during the rt-PA infusion and, finally, after reperfusion. The CPU activity in arterial and venous plasma increased two to three times during the rt-PA infusion in sham-operated control dogs. These animals had no copper coil, and consequently no LAD thrombus, but the surgical procedure will generate several small wounds leading to thrombin generation and systemic proCPU activation. It should be stressed, however, that there was no significant increase in CPU generation over the coronary vascular bed. A different picture emerged in the animals in which a thrombus was induced. The CPU activity in coronary venous and arterial plasma from one dog in the rt-PA-treated group is shown in Figure 14. The basal CPU activity in venous and arterial plasma was less than 2 U/L. There was a marked increase in CPU activity, both in venous and arterial plasma, when the copper coil was inserted, and a thrombus was formed within 30 to 45 minutes (experimental time). This increase in CPU activity continued for a while in venous blood but levelled off after 50–60 minutes

(experimental time). This "levelling off" effect was even more pronounced in arterial blood, where even a short period of reduced CPU activity was observed. A new burst of CPU activity occurred between 60 to 80 minutes when the rt-PA infusion was initiated, and the thrombus started to lyse. The mean CPU concentration in all dogs during this period was 22.4±4.3 U/L and 6.5±0.9 U/L in venous and arterial plasma, respectively (n=10, $p<0.001$). After the rt-PA infusion was stopped, CPU activity started to decline and had returned to baseline levels in both venous and arterial plasma by the end of the experiment. Interestingly, the first burst of CPU generation coincided with the insertion of the copper coil and thrombus formation, and the second burst coincided with the rt-PA infusion and lysis of the clot when active, fibrin-bound thrombin was released from the thrombus. These results indicate that thrombin is involved in CPU generation in this dog model of coronary arterial thrombosis.

Figure 14 CPU activity in venous and arterial plasma during thrombolysis with rt-PA

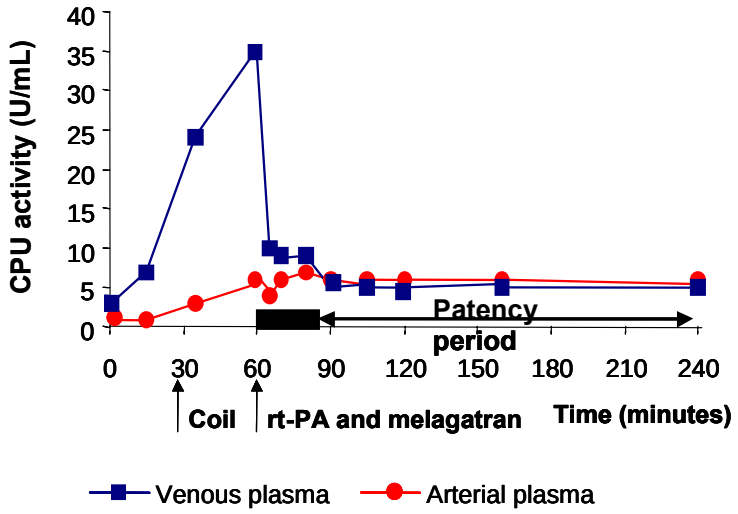


CPU activity in arterial and venous plasma from one dog in the rt-PA group.

To further support the hypothesis that thrombin generates CPU during thrombolytic treatment, a second group of 10 dogs received rt-PA in combination with a direct thrombin inhibitor (melagatran). CPU activity in arterial and venous plasma was analysed as above, and the results

from one dog in this group are illustrated in Figure 15. The basal CPU activity in this group was similar to that in the first group, and the activity increased, especially in venous plasma, in response to coil insertion and thrombus formation. However, after 60 minutes, when the infusion of the thrombin inhibitor was started, there was a drastic drop in CPU activity in venous plasma, and the increase in CPU activity in arterial plasma that was seen in-group 1 was also diminished. Control experiments also showed that melagatran had no inhibitory effect on CPU. These results strongly support the hypothesis that thrombin activates proCPU during thrombolytic treatment.

Figure 15 CPU activity in venous and arterial plasma during thrombolysis with rt-PA and a direct thrombin inhibitor.



CPU activity in arterial and venous plasma from one dog in the rt-PA-melagatran group.

In accordance with the results presented in Paper II, a significant shortening in the time to lysis and a longer patency period were also found in this study if rt-PA treatment was combined with a direct thrombin inhibitor. This means that inhibition of clot-bound thrombin may not be the sole explanation why direct thrombin inhibitors have a pro-fibrinolytic effect in this dog model of

arterial thrombosis. Inhibition of thrombin-mediated proCPU activetion could also facilitate fibrinolysis, as the thrombus will not undergo sufficient stabilisation in the absence of CPU. The theme of the third study in this series therefore became obvious: Would direct inhibition of CPU give the same results as inhibition of proCPU activation?

Thrombolysis and carboxypeptidase U inhibition – Paper IV

The experimental design of this study was identical to that in Paper III with the exception that the test compound MERGETPA, a direct inhibitor of CPU, was given before the copper coil was inserted and the thrombus formed. CPU is an enzyme that removes C-terminal lysine residues from partially degraded fibrin in a clot, and this process is initiated as soon as the thrombus has been formed. Thus, to achieve its maximum effect, a CPU inhibitor must be administered before thrombin is generated; as it can then immediately inhibit any CPU formed before it exerts its effect on fibrin.

Twenty dogs were randomised into 2 groups, one that received rt-PA, 1 mg/kg, as an intravenous infusion over 20 minutes, starting 30 minutes after thrombus formation, and one that received, in addition to rt-PA, the CPU inhibitor MERGETPA as an iv infusion starting 25 minutes prior to coronary artery occlusion. The primor study parameters in this study were time to lysis and patency time, which in the reference rt-PA group were 20 and 46 minutes, respectively, in similarity to those obtained in the previous study. The combination treatment with rt-PA and MERGETPA resulted in a significantly shorter lysis time, 15±1.5 minutes ($p<0.05$), and a longer patency time (87±16 minutes, $p<0.05$). However, all dogs in the rt-PA group re-occluded within 38±11 minutes while 7/10 dogs in the MERGETPA group reoccluded within 77±17 minutes. Although no thrombolytic treatment was given after the initial 20 minutes infusion of rt-PA, several of these re-occluded thrombi in the MERGETPA group lysed spontaneously. This indicates that a thrombus that is formed in the presence of a CPU inhibitor can easily be lysed by the endogenous fibrinolytic system in the dog. CPU inhibition may therefore be a suitable target for prevention of thrombus formation via facilitation of the endogenous fibrinolytic system.

The results from these studies in a dog model of arterial thrombosis have demonstrated that thrombin is involved not only in thrombus formation but also in its stabilisation via activation of proCPU. Inhibition of the stabilisation process can be achieved pharmacologically either by inhibition of thrombin, and thereby prevention of proCPU activation, or by direct inhibition of CPU. From our experiments, it is not possible to judge which of these treatment

regimens is most effective, but it can be hypothesised that CPU inhibition may be a safer treatment as it leaves the coagulation system intact.

The 1990s were the "golden era" for direct thrombin inhibitors, and several institutes and pharmaceutical companies had thrombin inhibitor programmes on their schedules. It is, however, not easy to directly compare the results from different studies with experimental coronary thrombosis. The techniques for inducing the thrombus have varied from study to study, and the experimental protocol concerning the mode of drug administration, dose levels, and imaging the thrombus and its lysis has also varied between studies [166-171].

Nevertheless, irrespective of the chemical nature of the thrombin inhibitor that has been used (polypeptides, tripeptides or peptidometics), our results from Papers II and III are consistent with those of other investigators in that thrombin inhibition in combination with rt-PA results in a shorter time to lysis and improved patency. The results from Paper II, that direct thrombin inhibitors in combination with rt-PA provide a significantly better protection from re-thrombosis than heparin, are also consistent with observations from other investigators [168].

An inducible carboxypeptidase activity has been demonstrated earlier in dogs with electrically induced coronary artery thrombosis and concomitant lysis with t-PA [165]. Some years later, other investigators [172,173] identified this carboxypeptidase activity as CPU, and they also showed that co-administration of t-PA and a CPU inhibitor; potato tuber carboxypeptidase inhibitor (PTCI), significantly improved t-PA-induced thrombolysis in a rabbit jugular vein thrombosis model.

Recently, it has also been shown that inhibition of CPU with PTCI results in a significantly reduced thrombus mass (45%) in the caval vein in mice after thrombus stimulation with ferric chloride [174]. Thus, inhibition of CPU is an antithrombotic therapy even in the absence of exogenous t-PA. There was, however, no effect of PTCI when the thrombus was induced in the carotid artery. All these studies are well in line with our findings in Paper IV, in which we showed that another inhibitor of CPU, MERGETPA, also facilitates rt-PA-induced thrombolysis in coronary arteries of the dog. In addition to this, we showed in Paper III that proCPU activation is most likely induced by thrombin, and that a small, direct thrombin inhibitor is able to inhibit this activation.

CONCLUSIONS

From the porcine studies we conclude:

- Activation of cardiac sympathetic nerves induces release of t-PA in the porcine coronary vascular bed. This release is independent of induction of tachycardia and local hyperaemia.

- The t-PA-response during sympathetic stimulation is, at least partially, mediated through stimulation of β-adrenergic receptors.

- Since prothrombotic mechanisms are activated at the systemic level during sympathetic activation, the t-PA response may constitute a local counter-regulatory mechanism for preventing the formation of occlusive intraluminal thrombi.

From the canine studies we conclude:

- That thrombin inhibition exerts a pro-fibrinolytic effect, and that a low-molecular weight direct thrombin inhibitor is more effective in inhibiting clot-associated thrombin than hirudin and heparin.

- Active CPU is produced locally in the coronary vascular bed during both thrombus formation and clot lysis.

- Administration of a small direct thrombin inhibitor attenuates this CPU generation, indicating that thrombin mediates the activation of proCPU into active CPU.

- Thrombin and/or CPU inhibition may be suitable targets for prevention of thrombus formation via facilitation of the endogenous fibrinolytic system.

FUTURE PERSPECTIVES

With regard to endogenous t-PA release as induced by sympathetic stimulation, it would be of importance to test the hypothesis that this mechanism is sufficient to induce thrombolysis in an experimental model. Another interesting approach may also be a combination of the approaches used in Paper I and Papers III-IV, *i.e.* to investigate whether local release of endogenous t-PA can lyse a thrombus if it is combined with an inhibitor of thrombin or CPU.

With regard to pharmacological thrombolysis, the dose of rt-PA has so far been the same in all studies. Suggestions for future studies may be a lowering of the rt-PA dose in combination with a thrombin- or CPU inhibitor aiming to find an optimal combination with respect to both efficacy and safety. Future studies of combination therapies with other novel thrombolytic agents are also of interest.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Blodproppsbildning (trombos) i blodkärl kan leda till en mer eller mindre uttalad avstängning av blodförsörjningen till livsviktiga organ såsom hjärta och hjärna vilket kan medföra förödande konsekvenser. Kroppen är därför utrustad med ett eget skydd för att motverka bildningen av sådana blodproppar. Detta sker genom att aktivera frisättning av proppupplösande substanser från blodkärlens innervägg. Den viktigaste av dessa är vävnadsplasminogenaktivator (t-PA - ”tissue-plasminogen activator”) som är nyckelenzymet för aktivering av proppupplösande mekanismer i blodkärl. Behandling med läkemedel bestående av rekombinant framställt t-PA (rt-PA) är idag en väldokumenterad terapi vid akut hjärtinfarkt.

Det är känt att aktivering av det sympatiska nervsystemet kan underlätta blodproppsbildning genom att förkorta blodets levringstid. Å andra sidan har det också visats att sympatisk aktivering, som t ex vid fysisk ansträngning eller stress, leder till stigande plasmahalter av t-PA. En ökning av mängden cirkulerande t-PA kan också åstadkommas med intravenös tillförsel av läkemedel med sympatikusliknande effekter. Om en aktivering av det sympatiska nervsystemet också ger en omedelbar t-PA-frisättning i hjärtats kranskärl är inte undersökt.

Läkemedelsbehandling med rt-PA har visat sig ha vissa svagheter i form av en relativt långsam öppning av blodkärlet och ibland tidig återuppbyggnad av blodproppen. Trombin är det enzym i blodet som ger upphov till bildning av blodproppar genom att omvandla det lösliga proteinet fibrinogen, till fibrin i blodproppen. Studier av innehållet i blodproppar från människa har visat sig innehålla en stor mängd trombin. En teori är att mekanismen bakom en tidig återkomst av blodproppen är att blodproppsupplösande behandling leder till en frisättning av trombin från blodproppen.

Detta trombin som finns inuti blodproppen är skyddat från inaktivering av trombinhämmare. Effekten av små trombinhämmare, som kan tränga in i blodproppen och därmed oskadliggöra trombin, kan därför tänkas ha en mer gynnsam effekt, än stora bulkiga hämmare (hirudin) och indirekta hämmare (heparin), som inaktiverar både fritt och fibrinbundet trombin i lika grad. Carboxypeptidase U (CPU) är ett kroppseget enzym som deltar i regleringen av kroppens system för att upplösa blodproppar, detta för att inte sårskorpan ska upplösas i förtid och därigenom orsaka blödning. Detta sker genom att minska bindningen av t-PA till blodproppens yta. Förstadiet till CPU (proCPU) cirkulerar normalt i plasma och aktiveras vid behov av trombin. En hämning av denna aktivering, antingen indirekt med en trombinhämmare eller direkt med en CPU-hämmare, skulle kunna ha en gynnsam effekt vid blodproppsupplösande behandling.

Frågeställningar

1) Ger en aktivering av det sympatiska nervsystemet upphov till en lokal t-PA-frisättning i hjärtats kranskärl? Om så är fallet, vilka är mekanismerna bakom denna stimulering?

2) Kan man genom att hämma både fritt och fibrinbundet trombin påskynda t-PA-inducerad blodproppsupplösning och förhindra återbildning av blodproppen?

3) Kan det trombin som frisätts under rt-PA-stimulerad blodproppsupplösning aktivera proCPU till sin aktiva form och kan i så fall en hämning av fibrinbundet trombin förhindra denna aktivering *in vivo*?

4) Har en direkt enzymhämning av CPU samma effekt som en hämning av CPU-aktiveringen med en trombinhämmare?

Metoder

Delarbete I utfördes på sövda grisar. De sympatiska nervfibrerna till hjärtat stimulerades elektriskt vid två frekvenser (1 och 8 Hz). Efter friläggning av hjärtat anlades en förbindelse (shunt) mellan den ven som dränerar blod från det främre nedåtstigande kranskärlets försörjningsområde och höger förmak. Prover för t-PA-analys togs samtidigt från stora kroppspulsådern och shunten före, under och efter sympatikusstimulering. I de uppföljande mekanistiska delstudierna studerades effekten av ökning av hjärtfrekvensen med en pacemaker liksom effekterna av kärlvidgning samt stimulering av olika sympatikusreceptorer genom lokal tillförsel av receptorstimulerande substanser.

Delarbete II-IV baserades på studier i en experimentell blodproppsmodell i kranskärlen på sövd hund. En kopparspiral fördes under genomlysning in i det främre nedåtstigande kranskärlet. Med denna modell bildas en tilltäppande blodpropp i kopparspiralen inom 3-5 minuter. Graden av avstängning skattades genom att kontinuerligt mäta blodflödet i kranskärlet strax nedan spiralen. I delarbete II studerades effekten av rt-PA ensamt och i kombination med direkta (melagatran, hirudin) eller indirekta (heparin) trombinhämmare. Tiden till proppupplösning och återbildande av blodpropp samt tid för öppetstående (patency) av kärlet registrerades. Aktivering av CPU vid blodproppsbildning och efterföljande upplösning med rt-PA med och utan trombinhämmare studerades i delarbete III. CPU-aktivitet mättes i samtidigt tagna arteriella och venösa kranskärlsprover. I delarbete IV studerades effekten av en direkt CPU hämmare (MERGETPA) som tillfördes cirkulationen innan kopparspiralen lades på plats, detta för att uppnå en maximal förebyggande effekt.

Resultat

Stimulering av hjärtats sympatiska nerver resulterade i en frekvensberoende massiv frisättning av både totalt och aktivt t-PA i kranskärlen. Varken hjärtfrekvensökningen eller kärlvidgningen *i sig* gav upphov till någon t-PA-frisättning. Lokal simulering av så kallade β-receptorer med isoprenalin gav en koronar t-PA-frisättning av lägre magnitud än den som noterades vid sympatikusaktivering, medan stimulering av så kallade α-receptorer inte hade någon signifikant effekt på t-PA-frisättningen.

En direkt lågmolekylär trombinhämmare med förmåga att hämma både fritt och fibrinbundet trombin (melagatran) gav den bästa behandlingseffekten i kombination med rt-PA (snabbare proppupplösning samt senare återbildande av blodproppen). En positiv effekt av hirudin erhölls först vid hög dos och effekten av heparin var också mindre påtaglig, vilket indikerar att hämning av fibrinbundet trombin är av stor betydelse i denna modell. Den lokala CPU-aktiviteten över hjärtats kärlbädd ökade markant i samband med trombinbildning och utvecklingen av en blodpropp runt kopparspiralen. En ytterligare ökning av CPU-bildningen erhölls när blodproppen löstes upp med rt-PA och fibrinbundet trombin frigjordes. En CPU-bildning erhölls således i samband med bildande eller frisättning av trombin. Resultatet ger stöd för hypotesen att trombin aktiverar proCPU både under blodproppens bildning samt under dess upplösning med rt-PA. Vid kombinationsterapi med rt-PA och en direkt trombinhämmare uteblev däremot den lokala CPU-bildningen i hjärtats kärl, vilket ger ytterligare stöd för denna hypotes.

I överensstämmelse med resultaten i delarbete II noterades även en signifikant tidigare proppupplösning och förlängning av perioden under vilken kärlet var öppet när rt-PA kombinerades med melagatran. Även en direkt CPU-hämning gav tidigare blodproppupplösning och senare återbildning av blodproppen jämfört med kontrollgruppen.

Slutsatser

Den kroppsegna förmågan att frisätta t-PA i hjärtats kranskärl vid aktivering av det sympatiska nervsystemet är mycket hög. Detta kan utgöra en lokal skyddsmekanism för att skydda mot den ökade benägenhet för blodproppsbildning som stress utlöser.

Kombination av rt-PA med en lågmolekylär trombinhämmare och/eller CPU-hämmare förstärker effekter vid blodpropp i kranskärlen och ger snabbare blodproppsupplösning och mindre tendens till återbildning av blodproppen.

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