Epigenetic influence on cardiovascular protective mechanisms in vivo: explorations of t-PA release and extracellular vesicle genetic content

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To My Family

Abstract

Background
Ischemic heart disease is one of the leading causes of death globally. This thesis explores endogenous mechanisms protecting against myocardial ischemia in context of epigenetics (changes in gene activity not caused by changes in DNA sequences). Epigenetic regulation of vascular thromboprotective mechanism was assessed, as well as the capacity of extracellular vesicle (EV) involvement in mediating epigenetic changes related to cardioprotection in ischemic preconditioning (IPC).

Aims
The aim of Papers I and II was to evaluate if histone deacetylase inhibition, by valproic acid (VPA) treatment, increases stimulated tissue plasminogen activator (t-PA) release capacity and affects plasminogen activator inhibitor-1 (PAI-1) levels *in vivo*, in healthy large animals and in an atherosclerotic cohort. The aim of Papers III and IV was to assess if coronary venous EV genetic content is affected by myocardial IPC *in vivo.*

Methods
In a porcine myocardial ischemia model transcoronary t-PA release was measured and compared between VPA treated (n=12) and untreated animals (n=10). In the clinical cross-over study (n=16), the perfused forearm model was used to measure single and repeated t-PA release capacity by isoprenaline provocation with and without VPA. PAI-1 was also measured. In a porcine model, EV were collected from coronary venous blood before and after myocardial IPC. The EV were isolated by differential ultracentrifugation and the preparation was evaluated by western blot, electron microscopy and nanoparticle tracking analysis. Changes in EV genetic content after IPC were identified by microarray and DNA sequencing.

Results
Animals treated with VPA demonstrated a significantly higher cumulative transcoronary t-PA release compared to controls. In the clinical study, VPA treatment resulted in increased cumulative t-PA release capacity during repeated isoprenaline stimulation, though there was no difference when comparing single stimulation sequences. Levels of PAI-1 were reduced after VPA treatment. Among 11678 mRNA sequences detected in EV, about 10% were up or down regulated after IPC. Among these, over half were increased, including several with association to cardioprotection and IPC. DNA fragments, representing all porcine chromosomes, were identified in EV. The DNA content in EV changed after myocardial IPC.

Conclusions
Intervention of HDACi, by VPA treatment, may improve actions of the fibrinolytic system by enhancing t-PA release capacity and reducing PAI-1 levels *in vivo.* In a future perspective, this may have clinical relevance as novel means of preventive strategies for ischemic heart disease. Myocardial IPC influences the composition of EV genetic content, including increases in gene transcripts associated to cardioprotecion. This may reflect a biological relevance of EV in delivering cardioprotective signals in IPC, although further studies are necessary to confirm such connection.

Keywords: myocardial ischemia, epigenetics, histone deacetylase inhibition, t-PA, extracellular vesicles, ischemic preconditioning

List of papers

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

I. Svennerholm K, Bergh N, Larsson P, Jern S, Johansson G, Biber B, Haney M. **Histone deacetylase inhibitor treatment increases coronary t-PA release in a porcine ischemia model.** *PLoS One*. 2014;9(5):e97260.

II. Svennerholm K, Haney M, Biber B, Ulfhammer E, Saluveer O, Larsson P, Omerovic E, Jern S, Bergh N. **Histone deacetylase inhibition enhances tissue plasminogen activator release capacity in atherosclerotic man.** *PLoS One*. 2015;10(3):e0121196.

III. Svennerholm K, Rodsand P, Hellman U, Lundholm M, Waldenström A, Biber B, Ronquist G, Haney M. **Myocardial ischemic preconditioning in a porcine model leads to rapid changes in cardiac extracellular vesicle messenger RNA content.** *Accepted for publication in International Journal of Cardiology, Heart & Vasculature*.

IV. Svennerholm K, Hellman U, Rodsand P, Lundholm M, Waldenström A, Biber B, Ronquist G, Haney M. **Coronary venous extracellular vesicle DNA content is altered by myocardial ischemic preconditioning in a porcine model.** *Manuscript*.

Summary in Swedish
Populärvetenskaplig sammanfattning

Ischemisk hjärtsjukdom (kranskärlssjuka) är en av de vanligaste dödsorsakerna i världen. Vanligen uppstår ischemi (syrebrist) i hjärtat till följd av en tromb (blodpropp) i hjärtats blodkärl. Fibrinolys, kroppens system att lösa upp tromber, skyddar kroppen mot att utveckla hjärtinfarkt. Hjärtmuskeln kan också öka sin tolerans mot syrebrist genom att hjärtat exponeras för korta upprepade perioder av ischemi, så kallad ischemisk prekonditionering (IPC).

Epigenetik handlar om hur en individs olika gener aktiveras eller tystas, vilket påverkar utveckling av egenskaper och sjukdom. Yttre faktorer såsom miljö, ålder och läkemedel kan skapa epigenetiska förändringar. Målsättningen med avhandlingen var att undersöka om man epigenetiskt kan förstärka uttrycket av tissue plasminogen activator (t-PA), nyckelenzymet som aktiverar fibrinolys, samt att studera epigenetiska signaler som kan vara hjärtskyddande vid IPC.

Patienter med ökad risk för ischemisk hjärtsjukdom har reducerad förmåga att frisätta t-PA. *In vitro* försök (cellkultur) har visat att t-PA genen står under epigenetisk reglering. Histondeacetylashämmare (HDACi) är en grupp epigenetiska substanser som ökar produktion och frisättning av t-PA. Det antiepileptiska läkemedlet valproinsyra (VPA) har visats ha HDACi-effekt och ökar t-PA *in vitro*.

I delarbete I studerades frisättning av t-PA hos sövd gris efter hjärtischemi (genom kortvarig avstängning av ett kranskärl). Försöksdjur som förbehandlats med VPA i en vecka visade sig ha betydligt högre t-PA frisättning än obehandlade djur, vilket talar för en ökad fibrinolytisk effekt.

I delarbete II undersöktes t-PA frisättning hos försökspersoner med underliggande kranskärlssjukdom. Frisättningen av t-PA från underarmens kärlbädd studerades under stimulering med isoprenalin i två konsekutiva omgångar. Försökspersonerna förbehandlades med VPA i fyra veckor och mätningarna utfördes vid två tillfällen, med respektive utan VPA. Varje försöksperson utgjorde således sin egen kontroll. Vi kunde inte påvisa någon effekt av VPA på t-PA frisättningen vid enstaka stimuleringar. Däremot resulterade VPA behandling i en signifikant högre t-PA frisättning vid upprepad stimulering. VPA behandling medförde också sänkta nivåer av plasminogen activator inhibitor-1 (PAI-1), den viktigaste hämmaren av t-PA, vilket indikerar ytterligare förstärkning av den fibinolytiska effekten.

Extracellulära vesiklar (EV) är mycket små membranförsedda partiklar som transporterar aktiva signaler mellan kroppens celler. De innehåller byggstenar (protein) och genetiskt material (DNA och RNA) som epigenetiskt kan reglera genuttryck och proteinproduktion hos mottagarceller.

I delarbete III och IV studerades huruvida IPC påverkar det genetiska materialet i EV isolerade från hjärtats cirkulation, och om sådana förändringar är associerade med hjärtskyddande effekter. På sövd gris framkallades IPC genom en snara runt ett kranskärl, och EV isolerades från blodprover tagna före och efter IPC. Därefter jämfördes det genetiska materialet. I delarbete III visades att 10% av RNA sekvenserna i EV ökade eller minskade efter IPC. Flera av de sekvenser som ökade har tidigare visats vara associerade med hjärtskyddande effekter. I delarbete IV kunde vi visa att EV innehåller DNA fragment som representerar alla grisens kromosomer samt att DNA innehållet förändrades efter IPC.

Sammanfattningsvis visar vi, för första gången, att HDACi kan ha en positiv behandlingseffekt på fibrinolys *in vivo*. Genom att farmakologiskt påverka kroppens t-PA frisättning finns potentiellt en möjlighet att i ett tidigt skede hämma trombutveckling och därmed minska risken för hjärtinfarkt och andra kardiovaskulära händelser. Våra resultat indikerar också att EV och dess genetiska innehåll kan ha en viktig biologisk skyddande effekt vid IPC och motiverar vidare studier.

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Abbreviations

ANOVA	Analysis of variance
AUC	Area under curve
BMI	Body Mass Index
C_A	Arterial t-PA concentration
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
C_{GCV}	Coronary venous t-PA concentration
CI	Confidence interval
CNS	Central nervous system
CONSORT	Consolidated standards of reporting trials
COX-2	Cyclooxygenase-2
CS	Coronary sinus
C_V	Coronary venous t-PA concentration
CVD	Cardiovascular disease
CVP	Central venous pressure
DNA	Deoxyribonucleic acid
ECG	Electrocardiography
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EudraCT	European union drug regulating authorities clinical trials
EV	Extracellular vesicles
FBF	Forearm blood flow
GcV	Great cardiac vein
GSK-3β	Glycogen synthase kinase 3 beta
HAT	Histone acetyltransferase
Hct	Hematocrit
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitors
HIF-1α	Hypoxia-inducible factor 1 alpha
Hs-CRP	High sensitive C-reactive protein
IABP	Invasive arterial blood pressure
IHD	Ischemic heart disease
IL-1β	Interleukin 1 beta
IPC	Ischemic preconditioning
I/R	Ischemia/reperfusion
ISEV	The International Society for Extracellular Vesicles
ISP	Isoprenaline
IV	Intravenous
LAD	Left anterior descending artery
Log 2 FC	Log 2 Fold Change
kg/m^2	Kilograms/square meters
min	minutes
miRNA	Micro ribonucleic acid
mL	milliliters
mRNA	Messenger ribonucleic acid
MV	Microvesicles
ng	nanograms
NGS	Next generation sequencing
nm	nanometer
PAI-1	Plasminogen activator inhibitor -1
PBS	Phosphate buffered saline
PGI_2/E_2	Prostagladin I2/E2
Q_{GCV}	Coronary venous blood flow
Riks-HIA	Register of information and knowledge about swedish heart intensive care admissions
RIPC	Remote ischemic preconditioning
RyR-2	Ryanodine receptor type 2
SC	Stem cells
SCAAR	Swedish coronary angiography and angioplasty registry
SD	Standard deviation
SEM	Standard error of the mean
STAT3	Signal transducer and activator of transcription-3
TCF/LEF	T-cell transcription factors/lymphoid-enhancer binding factor
TEG	Thromboelastography
TNF-α	Tumor necrosis factor alpha
t-PA	Tissue plasminogen activator
VPA	Valproic acid
WHO	World Health Organization

Introduction

Cardiovascular disease (CVD), and in particular thrombosis and ischemic heart disease (IHD), are the pivotal clinical problems on which this thesis is based. According to the World Health Organization (WHO), CVD is the number one cause of death globally. In 2012 an estimated 17.5 million people died from CVD where IHD was the major cause. More than 75% of global death of such etiology occurred in low and middle income countries, equally distributed among sexes (1). It is estimated that the number of deaths will increase and still remain the leading cause of death in 2030 (2).

Acute coronary thrombosis associated with rupture of an atherosclerotic plaque is the major cause of myocardial ischemia (3). When myocardial ischemia occurs, a number of factors decide whether or not it leads to tissue necrosis. In particular, it depends on the degree and duration of interruption in oxygen and nutrient delivery, as well as the metabolic activity of the heart (4). Also, and seemingly paradoxical, reperfusion of the ischemic myocardium is the phase where much of the tissue injury occurs. Reperfusion is associated with microvascular injury and an inflammatory condition with recruitment of inflammatory cells and mediators and local production of reactive oxygen radicals causing local tissue damage (5).

The overall aim of this thesis was to explore new aspects of endogenous cardiovascular protection mechanisms in the face of myocardial ischemia. In Papers I and II, the scientific question concerned how to enhance the fibrinolytic system, in context of dissolving coronary thrombosis. Mechanisms for increasing tolerance to coronary ischemia and limit reperfusion injury were assessed in Papers III and IV.

Epigenetics

Epigenetics concerns regulation of gene expression. It is an active and rapidly developing research area involving many different medical fields. Over the past two decades, the definition of “epigenetics” has changed. Initially, it was a broad term describing how genotypes might give rise to phenotypes during development, for example due to environmental exposures. A newer definition of epigenetics is the appearance of heritable changes in gene function that cannot be explained by changes in DNA sequences (6–8). There is still no widespread agreement in the scientific literature over a single definition, about which modulations that are considered true epigenetic mechanisms and whether heritability is required (9). Most agree that the term epigenetics refers to expression of genes that does not involve the DNA base-pair sequence, and that multiple mechanisms may influence gene transcription and

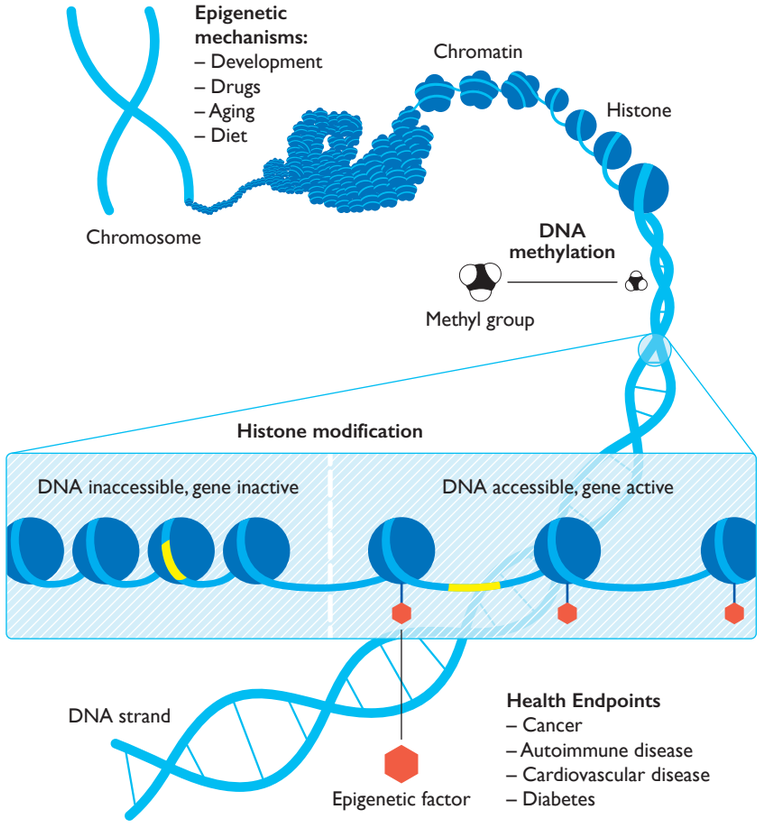

Figure 1. Epigenetic mechanisms. Several factors such as age, diet and drugs may cause epigenetic changes, for example DNA methylation and histone modifications, which may further affect health endpoints. The DNA is wrapped around histones and further packed to form the chromatin structure. Epigenetic changes affect the chromatin, and when it is unwrapped, DNA is accessible for transcription. Figure adapted from National Institutes of Health with permission from the publisher.

phenotype for the same genotype (6, 8). The main epigenetic features in humans include DNA methylation, histone modifications and RNA-based mechanisms (8). The epigenetic changes arise through a complex interplay between genome and environment, including factors such as age as well as exposures of smoking, drugs and diet (Figure 1). Epigenetic modulations are reversible and result in increased expression or silencing of genes. The epigenetic modifications control normal development, differentiation and growth, starting *in utero* and continuing through child- and adulthood (6, 8). Epigenetic mechanisms are also known to be involved in development of human diseases for example CVD and cancer, as well as chronic disorders such as metabolic syndrome, diabetes, obesity, and inflammatory disease (6).

The concept of epigenetics provides a new approach to understand regulation of gene expression and has been considered a paradigm shift in medical science and genetics. Additional understanding of epigenetics and its relevance and influence in developmental physiology, outcome and disease is needed in order to further use that physiological and pathophysiological knowledge to help designing new pharmacological therapeutic models. A more detailed presentation of epigenetics is beyond the scope of this thesis.

Epigenetics in cardiovascular physiology and disease

In cardiovascular medicine, epigenetic aspects have been associated with development and progression of CVD. This includes cardiac hypertrophy, heart failure, arrhythmias as well as influence on atherosclerosis illustrated in experimental studies (10). Epigenetic changes associated with cardiovascular risk factors such as smoking and diet are similar to epigenetic changes detected in patients suffering from CVD (11). A causal relationship of epigenetic changes leading predictably to CVD has not been demonstrated. Currently, there are no epigenetic contribution in CVD treatment, whereas a few epigenetic agents have been presented to bring treatment success within the field of cancer (10, 11). Epigenetic alterations may be future pharmacological targets in CVD. Therapeutic agents may prevent or reverse epigenetic changes contributing to CVD or alternatively, agents may generate epigenetic changes that improve the endogenous defense of CVD.

This thesis is based on two specific aspects of epigenetics (in the context of protection mechanisms against myocardial ischemia): histone acetylation and intercellular transfer of genetic content in extracellular vesicles (EV).

Epigenetics and histone acetylation

The first epigenetic mechanism studied in this thesis is modification of histones by altering their degree of acetylation. The chromosomal DNA is localized in the nucleus of the cell and tightly packaged around small positively charged proteins known as histones. These units form nucleosomes that together build the structure of chromatin (12) (Figure 2). Increased histone acetylation neutralizes their positive charges and consequently decreases the tight bindings of the DNA-histone complexes. The chromatin becomes less densely packed and is subsequently more accessible for transcription factors to initiate the process of transcription, resulting in increased gene expression. Two enzymes, histone acetyltransferase (HAT) and histone deacetylase (HDAC), regulate the degree of histone acetylation by transferring or removing acetyl groups from histones (12). By inhibiting or stimulating one or the other enzyme, the histones will become more or less acetylated leading to changes in expression of certain genes (Figure 2). Histone deacetylase inhibitors (HDACi) are a class of chemical compounds that inhibit HDAC. Currently, many HDACi are in clinical development, primarily for cancer treatment. A few have received approval for treatment of some specific hematological malignancies (13). Moreover, there is one clinically used drug, valproic acid (VPA), with other main pharmacodynamic effects and used for anticonvulsant treatment, which has later been discovered to also have HDACi activity (14).

Epigenetics and extracellular vesicles

The second aspect of epigenetics studied in this thesis concerns extracellular vesicles (EV). Extracellular vesicles are released from most cell types and they transport bioactive material such as proteins and nucleic acids to other cells in the body (15, 16). The contents (cargo) of EV may have functional effects in the recipient cell (the cell that receives and incorporates the EV) by epigenetic modifying its phenotype (17–21). These recent findings concerning EV and their ability to influence other cells have led to an entirely new perspective on intercellular communication.

Thesis overview

Papers I and II explore the influence of HDACi on endogenous fibrinolytic capacity *in vivo*, as a potential treatment in the setting of IHD and inadequate endogenous tissue plasminogen activator (t-PA) synthesis and release.

Papers III and IV assess changes in EV genetic content related to myocardial ischemic preconditioning (IPC) *in vivo*, in context of identifying potential epigenetic mechanism behind the state of increased myocardial ischemia tolerance.

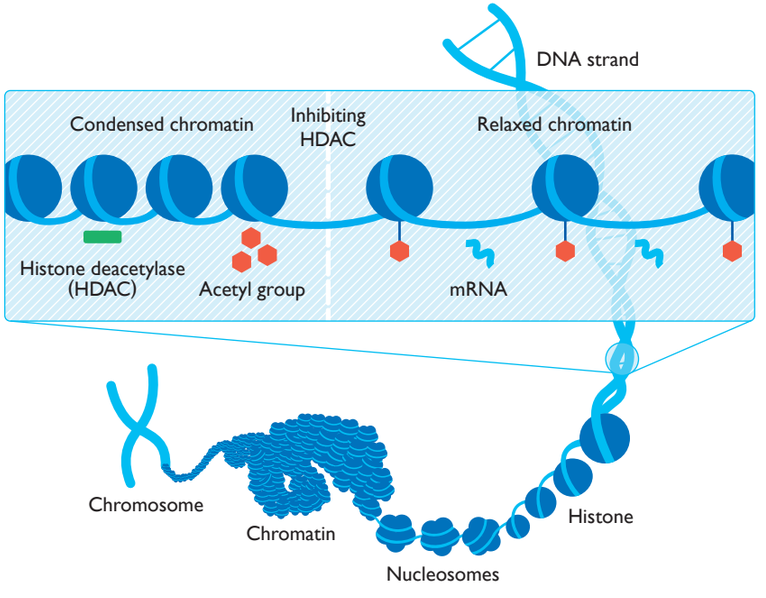

Figure 2. Chromatin structure and HDAC inhibition. The DNA strand is wrapped around histones and packed into nucleosomes that form chromatin, which in turn generate the chromosome. Histone acetylation is mediated by HDAC and HAT activity. The degree of acetylation influences the chromatin structure and subsequently gene transcription. By inhibiting HDAC, the chromatin is allowed to attain a relaxed configuration that results in enhanced gene transcription.

Thrombosis, fibrinolysis and tissue plasminogen activator

General background

Myocardial infarction and instable angina, together known as acute coronary syndrome, are most often caused by rupture of an atherosclerotic plaque in a coronary artery resulting in thrombus formation (3). If the thrombosis persists, it will result in irreversible injury and tissue necrosis, clinically known as myocardial infarction (4). The endothelium produces, stores, and releases t-PA. Active plasma t-PA is the key enzyme in the cascade of events leading to endogenous fibrinolysis. The enzyme catalyzes the conversion of the pro-enzyme plasminogen to its active proteolytical form plasmin, which is the core enzyme dissolving fibrin (22, 23) (Figure 3). The effect of local t-PA is greatly enhanced if it is present from start of the thrombus formation rather than after clot stabilization (24, 25). In addition, fibrin presence significantly increases the action of t-PA, which makes the enzyme relatively ineffective when there is no thrombosis present (26). Clearance of t-PA is mainly hepatic (27). Since its plasma half-time is short (minutes) and plasma t-PA concentrations are sensitive to hemodynamic changes and influenced by other systemic stimuli, it is not reliable to use changes in systemic plasma levels of t-PA as a marker of local t-PA release. Instead, by using regional perfusion studies, it is possible to assess local capacity for acute t-PA release (22, 28). Plasminogen activator inhibitor-1 (PAI-1) is the main inhibitor of t-PA, and increased fibrinolytic activity can be achieved either by increasing the amount of t-PA or by decreasing the levels of PAI-1 (29, 30).

Release of t-PA

The release capacity of t-PA varies for different vascular beds (31). There is a continuous constitutive t-PA secretion from endothelial cells that may quickly change to increased facultative release when needed (22). During acute repeated endothelial stimulation, a decline in t-PA release responses has been demonstrated (32 – 34). The intracellular signaling pathway for t-PA secretion is not completely understood, but it is dependent on G-proteins and increased levels of intracellular calcium (Ca2+) or cAMP (23). The main stimulus of local t-PA release is a thrombotic event, though adrenergic agonists and local ischemia will also result in acute t-PA secretion (32, 35 – 40). In addition, several endogenous factors stimulate acute t-PA release, including substance P (considered the most potent known stimulant in humans), bradykinin and coagulation factors, especially factor Xa and thrombin (22, 41 – 43) (Figure 3).

In a thrombotic event, the acute release of t-PA rapidly dissolves the thrombosis in order to re-establish local blood flow, letting oxygen and nutrients reach the ischemic area to prevent or limit tissue damage. The local plasma concentration of t-PA present during clotting (the first amount of t-PA released after endothelial stimulation) is very important in preventing the thrombus from becoming stabilized (24, 25).

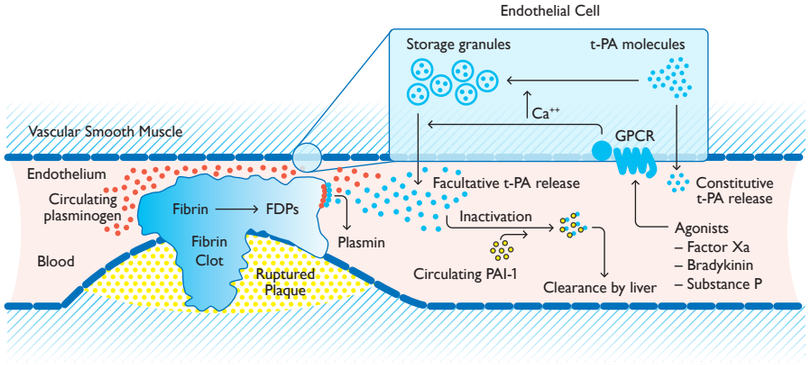

Figure 3. Schematic illustration of intravascular fibrinolysis. There is a constitutive t-PA release (continuous) from endothelial cells. The t-PA storage is secreted in response to the thrombus (fibrin clot) or by specific agonists, acting via the G-protein coupled receptor (GPCR). Free t-PA converts plasminogen to plasmin, which degrades fibrin into fibrin degradation products (FDPs). The t-PA is inactivated by PAI-1. Figure adapted from *Oliver et al.* (22) with permission from the publisher.

Deficient release of t-PA

While rapid local clot formation is vital for survival when bleeding, it is essential to have a responsive and functional fibrinolytic system to limit unnecessary and catastrophic systemic spread of thrombosis.

There are genetic variations of the t-PA locus that have been associated to diminished t-PA release (44). Further, a genetic low capacity for t-PA release has been demonstrated to increase the risk for major adverse cardiovascular events (45, 46). Reduced t-PA release has also been demonstrated in patients with well-known risk factors for CVD, such as hypertension, coronary atherosclerosis, obesity, chronic renal failure, and among smokers (47 – 51). Moreover, in patients with coronary heart disease, diminished t-PA release has been suggested to predict future risk of atherothrombotic events (52). The increased cardiovascular risk associated with inflammatory conditions may be related to the fact that the pro-inflammatory cytokines TNF-α and IL-1β are potent suppressors of t-PA expression (53, 54). In addition, elevated CRP levels, identified as a strong predictor of future cardiovascular events, have been shown to be associated with reduced endothelial t-PA release capacity in adults (55, 56).

Treatment perspectives of enhanced fibrinolysis

Current pharmacological CVD prevention concerning anticoagulation usually targets inhibition of platelets or function, or synthesis of coagulation factors (3). Despite advances in preventing and treating CVD, it still remains the leading cause of death worldwide, indicating that new clinical treatment strategies are needed. There is no pharmacological treatment that targets induction of the endogenous fibrinolytic system. Recombinant t-PA is sometimes used as an early acute treatment for stroke, myocardial infarction and pulmonary embolism (3, 57). This thrombolytic therapy is given first when patients present acute symptoms due to an already established thrombosis. Hence, the t-PA effect will be less potent (24, 25, 58). Recombinant t-PA is given intravenously (iv), which confers risk for serious bleeding complications also in organs that may not be affected by the thrombosis. In contrast to recombinant t-PA treatment, the endogenous fibrinolytic system is activated immediately when required. In addition, since t-PA concentration is increased only at the site of thrombus formation, the fibrinolytic effects are localized to where they are needed and consequently less dangerous for the rest of the body. Therefore, it is very attractive to find a pharmacological means to increase endogenous t-PA synthesis and release capacity.

Epigenetic regulation of t-PA expression

It has been shown that the t-PA gene is under epigenetic control. *In vitro* studies demonstrate that increased histone acetylation at the promoter region of the t-PA gene induces its expression (59–61). Increased degree of histone acetylation can be achieved by inhibiting the enzymatic activity of HDAC using HDACi. The well-established anticonvulsant and mood-stabilizing drug VPA has HDACi effects. This has been demonstrated both *in vitro* in different cell cultures, and *in vivo,* in animal and human models (14, 62–66). Recently, cultured human endothelial cells exposed to VPA showed a rapid dramatic dose-dependent increase in t-PA production (both mRNA and protein levels) (67). This *in vitro* observation of VPA enhancing t-PA expression formed the basis for Papers I and II: to test this hypothesis *in vivo,* in an animal model as well as in a human study.

Extracellular vesicles

Biogenesis, composition and function

Extracellular vesicles are nano-sized particles and cell products that can be detected in most body fluids. They are enclosed by bi-layered lipid membranes, which protect their contents from enzymatic degradation in the circulation. Extracellular vesicles are released from most cell types, including cardiomyocytes, and may exert both paracrine, or by entering the circulation, endocrine effects (15, 16, 68). The term EV includes different secreted membrane-enclosed vesicles such as exosomes and microvesicles (MV) (69). Currently, there is no consensus concerning the nomenclature, which is often inconsistent and incorrect. “Exosomes” are mostly used to designate any type of EV (70). Many publications describe that exosomes originate from endosomal compartments such as multivesicular bodies, and are generated by exocytosis when these compartments fuse with the cell membrane. On the other hand, MV are secreted through budding from the cell membrane (Figure 4). It is also common to separate EV according to their characteristics such as size, density and protein markers (15, 16). Currently, there are no specific markers that distinguish subsets of EV from one another and available isolation methods do generally result in a heterogeneous mixture of EV (70). Therefore, the studies of this thesis will invariably refer to all vesicles as EV. When discussing previous original works where other terms were used, EV will be used in order to have consistent nomenclature in this thesis.

Certain membrane proteins are enriched in EV, where the tetraspanins are some of the most characteristically associated, including CD9, CD63 and CD81 (16, 69, 71). The proteins may mediate cellular uptake of EV, and possibly also help EV to find their specific target cell types (72). The cargo of EV consists of cellular components such as bioactive lipids, proteins, messenger RNA (mRNA), micro RNA (miRNA) and most recently discovered but still debated, DNA (16, 18, 73). The EV content may be released in the cytosol or nucleus of the target cell and further change its phenotype (17–19). This was first illustrated when mice EV containing mRNA were transferred to human cells, and the mRNA was further translated into functional mouse proteins by the human recipient cells (17).

The sorting process of nucleic acid and proteins into EV is still not completely understood, but appears to be regulated (74). It has been shown that environmental effects on the parental cells can alter their EV genetic content. Extracellular vesicles secreted from mouse cells exposed to oxidative stress had a different mRNA content compared to EV from controls. Further, the stress-derived EV conferred resistance against oxidative stress to recipient cells (75). Another group showed that cellular stress caused by hypoxia or TNF-α influenced both protein and mRNA content in EV from human endothelial cells (76). One possible biological implication of this phenomenon might be that parental cells may dynamically compose and change EV content in order to produce tailor-made functional EV. These EV could convey signals, including emergency survival signals, to other cells during environmental threats such as stress and hypoxia.

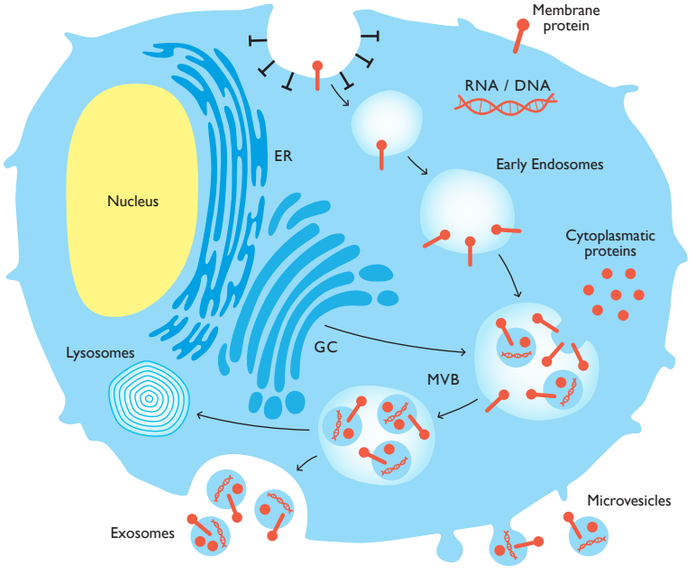

Figure 4. Schematic design of EV biogenesis. Exosomes are generated and released from the cell when multivesicular bodies (MVB) fuse with the plasma membrane while microvesicles are defined as budding from the plasma membrane. The vesicles carry RNA/DNA and proteins from the cytosol, Golgi complex (GC) and cell membrane. Abbreviation in figure: ER = Endoplasmatic reticulum. Figure adapted from *Waldenström et al.* (77) with permission from the publisher.

Extracellular vesicles and DNA

Currently, there is a discussion within the EV research field concerning whether or not DNA actually does exists in EV. Some findings have indicated that EV contain RNA but no DNA (17), while there are *in vitro* and *in vivo* findings that support the existence of genomic DNA in EV from various cell types. Mitochondrial DNA has also been found in EV (78). Some of the first reports identifying EV DNA fragments were made on EV from human prostate cells (79–81). Consistent with these findings, others demonstrated DNA in EV released from glioblastoma cells in culture and rat tumor cells *in vivo* (82). In a study on EV from murine cardiomyocytes, the identified EV DNA fragments were also transferable to the cytosol and nucleus of cultured fibroblasts (18). More recently, EV from a broad panel of cancer cell lines, as well as EV from plasma of patients with pancreatic cancer, have been shown to contain DNA fragments representing the whole genome, including the mutation of the cellular tumor source (73, 83). Plasma-derived EV from healthy patients have also been demonstrated to contain DNA (84).

The functional impact of EV RNA is being studied, though the biological role of EV DNA transfer as well as dynamics in EV DNA content is less explored. While working on Papers III and IV, new reports became available showing that EV from human plasma and cultured cells contained DNA fragments that were transferable and detected in the nucleus of recipient cells. A physiological significance was further demonstrated when the specific EV DNA was associated with increased DNA transcription, gene expression and protein content, along with influence on the function of recipient cells, both *in vitro* and *in vivo* (19, 85). A biological functional impact of EV DNA was also presented in another study where rat epithelial cells transformed by human oncogenes resulted in release of EV containing DNA covering the entire rat genome, including the human oncogene. When non-transformed cells internalized these EV, it led to cellular proliferation; thus indicating a functional aspect of the EV DNA (86).

Extracellular vesicles within the cardiovascular field

In 2007, it was demonstrated that cardiomyocytes release EV (68). Later, it was shown that EV from cultured murine cardiomyocytes contained DNA and mRNA. When these EV were added to a fibroblast culture, the nucleic acid could be detected in the fibroblasts nucleus. The transfection changed expression of hundreds of genes, resulting in phenotypic alterations of the recipient fibroblasts (18). An *in vitro* experiment demonstrated that variation in the metabolic environment of cardiomyocytes, by using different growth factors, changed the composition of mRNA in secreted EV (87). Concurrently, others showed that EV from hypoxic cardiac cells contained a different set up of proteins compared to EV from controls (88).

When stem cells (SC) first demonstrated signs of having potential in cardiac regeneration and improved cardiac function, it was proposed that they differentiated into new cardiomyocytes. Recently, it was suggested that part of the cardioprotective effect was strongly associated to paracrine signals like EV (16). In murine myocardial ischemia/reperfusion (I/R) models, reduced myocardial infarct size was shown when EV, isolated from human mesenchymal SC as well as from murine cardiac progenitor cells, were administrated after ischemia before reperfusion (89, 90). The EV treatment effect seemed to be caused by activation of pro-survival pathways and reduced oxidative stress in the I/R heart (91). Most recently, human cardiac progenitor-derived EV

conferred reduced cardiomyocyte apoptosis as well as functional improvement in *in vivo* infarcted mice hearts previously thought to be permanently damaged (92, 93). The studies have been confined to cardioprotective aspects of miRNA, while studies concerning fragments of mRNA or DNA have not been published.

Extracellular vesicles in ischemic preconditioning

Ischemic preconditioning (IPC) is a phenomenon of endogenous cardioprotection through adaptive tolerance of myocardial ischemia. This was described in 1986 and was shown to be a very potent form of normothermic myocardial protection (94). Ischemic preconditioning involves brief (minutes) and repeated periods of myocardial ischemia, too short to cause permanent injury by themselves, each followed by a short phase of reperfusion. The treatment leads to an increased cellular tolerance for ischemia and is measured by reduction of infarct size during a subsequent prolonged myocardial ischemic period (94). The defense has two phases: one immediate transient (two – three hours) and one delayed phase, apparent 12 – 24 hours later, with effects lasting up to four days (95). The protective effects of IPC have been reproduced in several species including humans, though it has not yet been confirmed and implemented as a clinical treatment (96). While consistent potent protective effects have been shown in large animal models, so far the same effects have been difficult to consistently demonstrate in clinical studies. A similar degree of cardioprotection has been shown with short ischemic episodes in remote organs distant from the heart, known as remote IPC (RIPC) (97, 98). Also, protective effects have been shown if short ischemic cycles are performed after a prolonged ischemic event, called ischemic post-conditioning (99).

Many possible cellular molecular pathways related to ischemic injury mitigated by IPC have been demonstrated, but the actual protection mechanisms are not yet fully understood. Ischemic preconditioning generates a variety of chemical signals and metabolites that alert the myocardium of impending danger and trigger complex signaling cascades that convert cells to a defensive phenotype. Several factors have been suggested and seem to be involved; such as adenosine, bradykinin, prostaglandins, nitric oxide, Ca^{2+} and reactive oxygen species. Also, the mitochondria appear to be a central actor in early cell recovery or demise (95, 96).

As previously described, some studies have suggested EV as mediating cardioprotection in ischemic or infarcted hearts (89, 90, 92, 93). Consistently, EV have been assessed in experiments of cardioprotection in IPC and RIPC. In a rat *ex vivo* model, coronary perfusate containing EV from IPC hearts decreased infarct size in ischemic hearts, similarly to cardioprotection afforded by IPC (100). In another report, EV from murine mesenchymal SC following IPC limited apoptosis when transferred to ischemic cardiomyocytes, and improved cardiac outcome when mobilized to *in vivo* infarcted murine hearts (101). The cardioprotective effects in this study, as well as in an experiment with RIPC after myocardial infarction, were suggested to be caused by specific miRNA enriched in EV due to IPC (101, 102). Other groups have shown in preliminary results that EV, isolated from plasma after RIPC (in humans and rats), protected the heart against I/R injury *in vitro, ex vivo* and *in vivo* (rat) (103, 104).

Even though this is a rapidly evolving research field, there is still limited knowledge about the importance and functional aspects of EV in cardiovascular physiology and pathology, as well as how they might protect the heart from ischemic injury. The discoveries of EV as cardioprotectors and the environmental influences of EV genetic content are the basis for the hypotheses in Papers III and IV.

Aim

The overall aim of this thesis was to explore aspects of epigenetic regulation affecting endogenous protective mechanisms against myocardial ischemia. Epigenetic regulation of vascular thromboprotective mechanism as well as the involvement of EV-carried genetic material related to protection in myocardial IPC were assessed.

The specific aims were:

- To evaluate if HDACi intervention, by VPA treatment, increases stimulated coronary t-PA release capacity in a healthy large animal model.
- To assess if VPA treatment increases t-PA release capacity after single and repeated stimulation in atherosclerotic subjects.
- To test if VPA treatment decreases PAI-1 levels in an atherosclerotic cohort.
- To explore if coronary venous EV mRNA content is affected by *in vivo* myocardial IPC.
- To identify DNA in porcine plasma-derived EV and to evaluate if coronary venous EV DNA is affected by *in vivo* myocardial IPC.

Materials and Methods

The work has been based on a multidisciplinary collaboration between the Sahlgrenska Academy at the University of Gothenburg (Department of Anesthesiology and Intensive Care and the Department of Molecular and Clinical Medicine), Umeå University (Department of Anesthesiology and Intensive Care Medicine and the Department of Cardiology) and Uppsala University (Department of Medical Chemistry).

The animal experiments (Papers I, III and IV) were performed at Umeå University, Umeå and the clinical study (Paper II) at Sahlgrenska University Hospital, Gothenburg.

Ethical considerations

The animal studies were performed following approval from the regional Animal Research Ethics Committee in Umeå (documents A123-10 and A182-12) and conducted in adherence with Guide for the Care and Use of Laboratory Animals (1996) from the National Academy of Science, USA. At the end of the experiments animals were euthanized by a bolus of pentobarbital and potassium chloride.

The clinical study was approved by the regional Human Research Ethical Board at the University of Gothenburg (document 935-12) and by the Medical Product Agency in Sweden (EudraCT nr: 2012-004950-27). The nature, purpose and potential risks of the study were presented both orally and in written form, to each individual before written informed consent was obtained. Participants were allowed to withdraw from the study at any time. The study was performed according to the principles of the Declaration of Helsinki.

Animal studies (Papers I, III and IV)

Animals

Three months old Swedish land-race pigs, about 40 kg of weight, supplied by the local school of agriculture in Umeå were used. All animals were brought to the laboratory one or two days (if not stated otherwise) before the experiment.

Paper I included 29 animals of which 22 were used for final analysis; 12 in the treated group and ten in the control group. The remaining seven animals were excluded due to anomalous coronary vasculature (which made it difficult to place the catheters and perform the study) or due to circulatory collapse related to post-ischemic malignant

arrhythmias. In addition, 34 pigs were used in the methodological development in an initial pilot study, where different alternatives for stimulation of t-PA release were explored.

Papers III and IV included six animals. One animal was excluded due to circulatory collapse during the IPC intervention. Five animals completed the protocol for EV sampling before and after myocardial IPC.

Anesthesia and surgical preparation

Animals were fasted 12 hours prior to the trials with free access of water. After intramuscular premedication with ketamine and xylazine, anesthesia was induced by a bolus of pentobarbital iv. Throughout the experiments, anesthesia was maintained by continuous infusions of pentobarbital, fentanyl and midazolam, without use of muscle relaxants. The animals were intubated transtracheally and mechanically ventilated to normoxia and normocapnia. During the experiments, animals received iv fluids. They were continuously monitored with electrocardiography (ECG), invasive arterial blood pressure (IABP), central venous pressure (CVP) and core body temperature was maintained at 38–39° C using an external warming pad.

A central venous catheter was placed in the internal jugular vein to measure CVP and for drug and fluid infusion. A small branch of the external carotid artery system was cannulated with a long catheter (its end placed in the descending aorta) for blood gases, IABP and aortic blood samples (Paper I). For the initial pilot study with intracoronary infusions of drugs, the common carotid artery was cannulated with an introducer, through which coronary arterial catheterization was accomplished. To sample coronary venous blood and measure coronary venous blood flow (Q_{GCV}, Paper I) a coronary sinus (CS) catheter was inserted through the jugular vein with the sampling tip in the proximal great cardiac vein (GcV) (myocardial venous drainage), using fluoroscopic guidance and measurements of venous oxygen saturation to confirm correct position. Pigs have a left azygos vein (systemic venous blood) draining into CS instead of the right hemiazygos draining into superior vena cava as in humans (105). The joining of the GcV and azygos vein becomes the CS. This anatomical aspect is essential to be aware of while placing the CS catheter. The correct placement of the catheter tip is as distal as possible in GcV (and not azygos), and is critical since we aimed to sample myocardial venous blood specifically. Through a midline sternotomy and pericardiotomy, a patched snare was placed around the middle part of the left anterior descending artery (LAD), not including corresponding vein (which would interrupt venous drainage) (Figure 5). After surgical preparation, heparin was given to prevent thrombosis on the catheters, and one hour of rest was allowed before baseline blood sampling.

In all animal studies, myocardial ischemia and accurate reperfusion sampling was confirmed by more than doubling of coronary lactate production, measured in GcV blood samples.

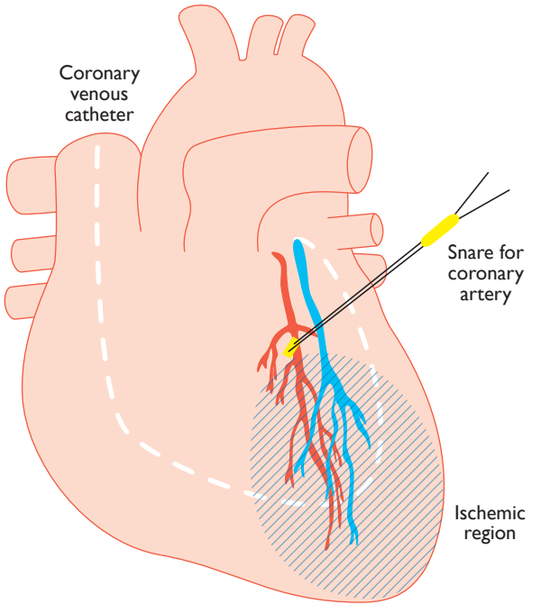

Figure 5. Schematic illustration of the surgical heart preparation (Papers I, III and IV). The LAD and corresponding vein are depicted. The coronary snare, when drawn, generates temporary ischemia in a specific and large part of the left ventricle. The CS catheter is introduced through the right atrium and CS in order to place the sampling tip in the proximal GcV that drains the ischemic area. Figure adapted from Paper I with permission from the publisher.

Study protocol and blood sampling (Paper I)

Animals in the intervention group (n=12) where brought to the laboratory one week before the trial for oral administration of VPA, (Ergenyl Retard, Sanofi Aventis, Sweden) 500 mg twice daily, until the day before the experiment. Animals in the control group (n=10) were brought to the laboratory one or two days before the experiment. Blood samples for VPA plasma concentration were taken after surgical preparation.

Coronary blood flow was assessed using retrograde thermodilution (106). Triplicate of baseline aortic and coronary venous blood samples for t-PA, followed by Q_{GCV} measurements, and single blood samples for PAI-1, were collected before the LAD snare was occluded for 10 min. Simultaneous aortic and coronary venous blood samples for t-PA, followed by Q_{GCV} measurements, were collected at min 1, 3, 5, 7 and 10 of reperfusion (36). Only static plasma levels of PAI-1 were assessed since previous studies have shown no net release of PAI-1 due to coronary ischemia (32).

Study protocol and blood sampling (Papers III and IV)

Baseline coronary venous blood samples (approximately 100 mL) for EV collection were drawn before IPC in all five animals. Myocardial IPC was created by temporary occluding the LAD snare for 10 min followed by 20 min of reperfusion, repeated in four cycles (107). Coronary venous blood samples for EV isolation (approximately 100 mL blood) were taken more or less continuously during a 20 min period starting 20 min after last LAD snare release.

Clinical study (Paper II)

Clinical subjects

Subjects where recruited from the Swedish national coronary angiographic and angioplasty register (Riks-HIA/SCAAR). Patients suffering from left main stenosis or 2- or 3-vessel disease, registered during 2010 and 2011 at the Sahlgrenska University Hospital, were identified from the register. Among approximately 1100 patients, women were excluded and patients were further ranged according to body mass index (BMI). Eighty potential male subjects with highest BMI (but less than 35 kg/m2) were invited to participate by mail, of whom 50% responded positively. Inclusion criteria were male patients, less than 85 years who had been treated for myocardial infarction at Sahlgrenska University Hospital more than one year ago. Smokers, BMI >35 kg/m^2, patients with symptomatic CVD or uncontrolled hypertension, anticoagulation therapy other than aspirin, medications interacting with VPA, chronic diseases contraindicated with VPA, malignancy, on-going infection, psychiatric disorder, alcoholism, epilepsy or if not able to understand study information were excluded. Due to exclusion criteria 17 individuals were left out. Thereby, 23 subjects were included and underwent basic physical examination, ECG and analysis of routine blood chemistry (blood cell counts, electrolytes, creatinine, liver enzymes, coagulation tests, glucose, and high sensitive C-reactive protein, hs-CRP). After inclusion, seven subjects dropped out for different reasons; without giving an explanation (n=3), difficulties cannulating vein or artery (n=2), vasovagal reaction (n=1) and gastrointestinal side effects during VPA treatment (n=1). A total of 16 subjects completed the trial and were included for analysis, although three subjects had three out of four successful measurements (due to problems with the venous cannula and mishandling of blood samples) (Figure 6).

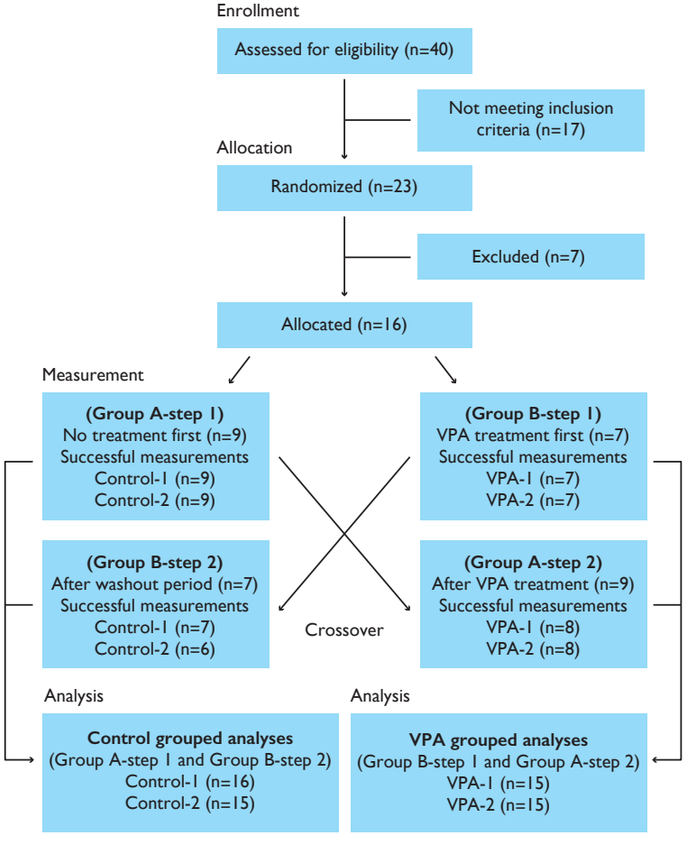

Figure 6. CONSORT flow diagram. After allocation and further exclusion, 16 subjects were placed in one of two groups. At first, Group A measured as a control while Group B was measured after VPA treatment. Next, Group A was measured after four weeks of VPA treatment and Group B was measured after a washout period (at least four weeks) without VPA, as a control. All measurements with VPA were pooled into one group, as well as control measurements were pooled into one group, during statistical analysis. Figure adapted from Paper II with permission from the publisher.

Study design

In this cross-over study, each subject was pharmacologically stimulated to increase t-PA release on two separate days; one day after VPA treatment and one day as a control measurement (without VPA). Identical study protocols were used on both days. To evaluate repeated t-PA release capacity, subjects were stimulated and measured twice on each day, with one hour of rest between each stimulation sequence (termed VPA-1 and VPA-2, control-1 and control-2). The subjects received oral VPA, (Ergenyl Retard, Sanofi Aventis, Sweden) 500 mg twice daily, during four weeks before VPA-1/VPA-2 were performed. The washout period was at least four weeks long before control-1/control-2 were performed. The 23 subjects that met the inclusion criteria were randomized (by lot) to either start with control measurements (Group A) or to start with VPA treatment measurements (Group B). Seven participants performed VPA-1/VPA-2 before control measurements, and nine subjects performed control-1/control-2 before VPA measurements (Figure 6). Prior to a test day, subjects were restricted from theophylline or caffeine containing food (bananas, coffee, tea, chocolate) for 12 hours, NSAID, alcohol and strong physical activity for 24 hours and vitamin substitution for the last ten days (108–112).

The perfused forearm model

Acute t-PA release capacity was evaluated using the forearm vascular bed and intra-arterial isoprenaline (ISP) (Hospira, USA) stimulation, as assessed with the perfused forearm model (113). The model included measurement of circumference and volume (by water displacement) of the forearm. Forearm blood flow (FBF, expressed in mL • min^{-1} • 100 mL^{-1} tissue) was measured by venous occlusion plethysmography (114). By briefly obstructing venous drainage, but not arterial inflow, forearm volume increases over time, proportional to FBF. A circumferential mercury-in-silastic strain gauge was used to measure increases in forearm circumference and thereafter derive volume changes. For arterial t-PA sampling as well as for the ISP infusion and IABP measurements, an arterial catheter was introduced in the brachial artery (under sterile conditions and mostly by ultrasound guidance). A venous cannula was placed in retrograde position, into an ipsilateral antecubital vein for venous t-PA sampling (Figure 7). The method is well-established, used for many years, by different research groups and in several studies for measuring acute t-PA release capacity (22, 28, 43, 52, 113). The technique is associated with a few limitations such as its sensitivity to discrete motions and less accuracy in obese patients.

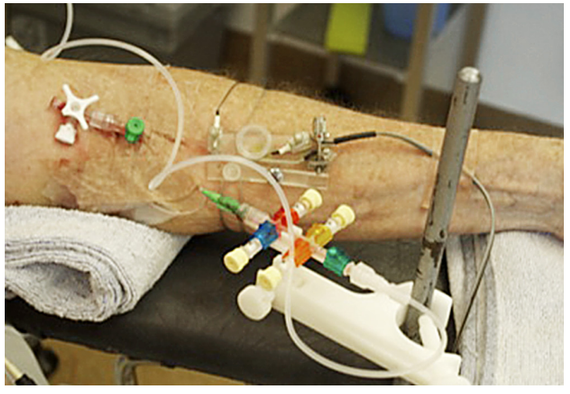

Figure 7. The perfused forearm model. An arterial line (for arterial t-PA sampling, ISP infusion, and IABP) and a venous cannula (for venous t-PA sampling) were placed in the antecubital fossa. The mercury-in-silastic strain gauge, used to measure forearm circumference was positioned right below, and the inflating cuff interrupting venous drainage, was placed around the upper arm (not seen in photo). The photo is taken from one of the study patients.

Study protocol and blood sampling

During the experiment, which was performed in a calm and quiet study room, subjects rested in a recline chair, continuously monitored with ECG and IABP. After cannulation, one hour of rest was allowed before baseline recordings. Blood samples for routine chemistry analysis, glucose, serum lipids, VPA plasma concentration, PAI-1 and thromboelastography (TEG; a test reflecting hemostasis and detection of dynamics in clot formation, stabilization and fibrinolysis) were taken. Arterial and venous baseline blood samples for t-PA analysis were collected in triplicates before ISP infusion. During the 20 min ISP stimulation phase (300 nanograms (ng)/min), venous blood samples for t-PA were collected at 1.5, 3, 6, 9, 12, 15 and 18 min. No arterial samples were obtained in order not to interrupt the ISP infusion; instead these values were interpolated from the arterial samples taken at baseline and at the end of the study. After each blood sampling, three to five venous occlusion sequences were taken to calculate mean FBF. Only static arterial and venous levels of PAI-1 were assessed, since previous studies show no net release of PAI-1 during forearm ISP stimulation (38).

Analyses and calculations of t-PA release (Papers I and II)

All blood samples were collected in chilled syringes and placed in tubes containing sodium citrate buffer. The samples were further held on an ice bath until being centrifuged as soon as possible. Plasma specimens were kept in -70° C until being thawed for further analysis.

t-PA and PAI-1 analyses

Total amount of plasma t-PA antigen was determined by enzyme-linked immunosorbent assay (ELISA) (TintElize and TriniLize t-PA antigen, Trinity Biotech). All samples were assayed in duplicate, and mean concentration was used for further calculation. The intra-assay coefficient of variation was <5%. Since there is no commercial porcine standard available, the assay in Paper I was calibrated using recombinant porcine t-PA diluted in porcine plasma.

Plasma levels of total PAI-1 antigen were determined by ELISA (Porcine PAI-1, Innovative Research and Technozym PAI-1, Haemochrome Diagnostica). Samples were assayed in duplicate, and the mean level was calculated. A mean of the arterial and venous PAI-1 level was calculated (Paper II).

Calculation of t-PA release and cumulative t-PA release capacity

Baseline and stimulated regional t-PA release (the t-PA flux at each point measurement) was calculated according to the Fick principle, as the product of veno-arterial t-PA concentration difference and local plasma flow, using the following formulas:

Paper I: **(CGCV – CA) • QGCV • ((101-Hct)/100)**
C_{GCV} = coronary venous t-PA concentration, C_A = arterial t-PA concentration,
Q_{GCV} = coronary venous blood flow, Hct = hematocrit

Paper II: **(CV – CA) • FBF • ((101-Hct)/100)**
C_V = venous t-PA concentration, C_A = arterial t-PA concentration,
FBF = forearm blood flow, Hct = hematocrit

Cumulative t-PA release was calculated by area under the curve (AUC), using t-PA fluxes on y-axis and time on x-axis (Figure 8).

In Paper II, measurements and calculations from all subjects from VPA-1 were grouped and the same was done with all data from VPA-2, control-1 and control-2. Grouping of data was done independently of whether VPA measurements were performed before or after control measurements (Figure 6).

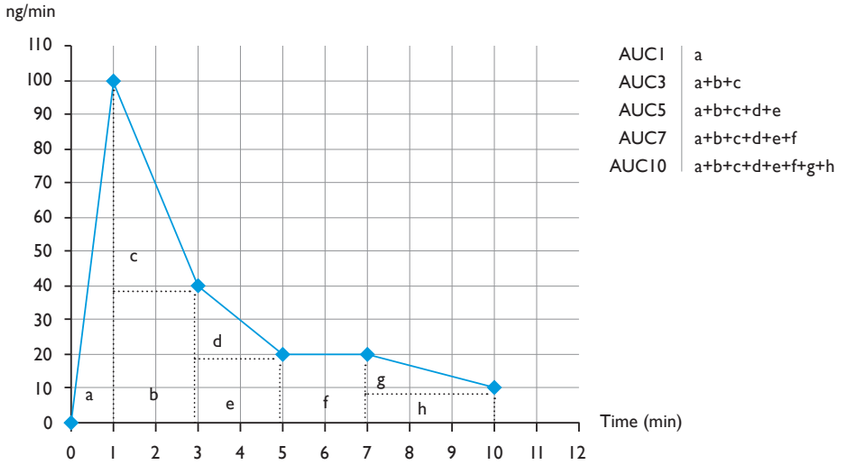

Figure 8. Calculation of cumulative t-PA release over time, area under the curve. The calculation is based on simple geometry, here using five point measurements. The t-PA fluxes are expressed on the y-axis and time on the x-axis. Abbreviations in figure: ng=nanograms, min=minutes, AUC=area under curve

Repeated stimulated t-PA release capacity (Paper II)

The influence of VPA on repeated stimulated t-PA release capacity, also termed exhaustion, was determined by comparing cumulative t-PA release from first and second stimulation sequences on the same day (control-2/control-1 and VPA-2/VPA-1). Ratios were first calculated for each individual, and then grouped before further statistical analysis.

Additional laboratory analyses

The analyses of VPA plasma concentration (Papers I and II) and routine blood tests (Paper II) were performed at the Department of Clinical Chemistry, Sahlgrenska University Hospital, Gothenburg. The TEG analyses (Paper II) were performed at the Intensive Care Department, Sahlgrenska University Hospital, Gothenburg.

Extracellular vesicles (Papers III and IV)

Isolation of extracellular vesicles

Coronary venous blood samples were immediately centrifuged to separate the plasma, which was further mixed with equal amounts of phosphate-buffered saline (PBS). Subsequently, differential ultracentrifugation (with sucrose density gradient-based

isolation) was used to isolate EV. The protocol involves a series of low-speed centrifugations to eliminate cells and cellular debris, followed by higher speeds ultracentrifugation to pellet the EV and remove proteins and larger vesicles as apoptotic bodies (115). Between the first and second steps of ultracentrifugation, the EV pellet is exposed to nuclease digestion to remove any DNA or RNA located exterior to the vesicles. The pellet is loaded on a sucrose gradient before the second ultracentrifugation, to further achieve separation of contaminating material such as proteins (EV are efficiently isolated according to their density) (115, 116). The EV fractions are collected by taking 2–3 mL of solution from the gradient zone. Then, fractions are washed with PBS before the last (third) ultracentrifugation. The final EV pellet is diluted in PBS before being stored in –80° C until further preparation.

Evaluation of extracellular vesicle preparation

Currently, it is a challenge to obtain a pure EV preparation. Therefore, it is necessary to assess and demonstrate the quality of the EV preparation by confirming absence of potential contaminants as apoptotic bodies and cells. Furthermore, the isolated vesicles need to be characterized. The EV evaluation can be achieved by western blot together with electron microscopy (EM) and nanoparticle tracking analysis (NTA) (70, 115, 117). Western blot may characterize, but not quantify, isolated particles as EV and identify potential presence of apoptotic bodies or cells by using specific protein markers (115). Recently, it was suggested that one should quantify several proteins in an EV preparation (70). In our studies, we were restricted to the use of only two proteins due to limited amount of commercially available antibodies for porcine antigens. CD81 was used as EV marker and GRP78 as a negative control to detect potential contamination of cells and apoptotic bodies. Western blot was performed on lysed EV and on crushed porcine myocardial tissue in cell suspension. The EM analysis characterizes and determines the morphology of single isolated vesicles. It was performed at the EM unit Emil, Clinical Research Center in Stockholm. The size of isolated EV as well as the EV amount was measured for each animal, before and after IPC, using NTA.

Extracellular vesicles – DNA/RNA extraction

The extraction of DNA and RNA from EV was performed by a DNA/RNA preparation kit according to the manufacture's instructions and stored in –80° C. The extracted RNA and DNA were quantified using a nanodrop spectrophotometer.

Microarray – gene expression analysis (Paper III)

Microarray technology was applied to quantify and characterize the changes of mRNA content in EV before and after IPC. In a gene expression microarray, all mRNA sequences in the sample are evaluated simultaneously. The amount of each mRNA sequence reflects the transcription activity of each specific gene. The experiments are complex and produce large amounts of complicated data, difficult to interpret without specific bioinformatical consultation (118). In our study, the microarray assays were performed at a laboratory specialized in the technology, at the Array and Analysis Facility, at Uppsala University. The microarray data was normalized, validated and statistically filtered using specific software packages provided by Affymetrix and the Bioconductor project (www.bioconductor.org).

The average expression of each gene was calculated as the mean value of samples before IPC (n=5) and the mean of the samples after IPC (n=4, one was excluded due to lack of enough material for analysis). The levels for significant detection as well as significant up or down regulation were set according to the bioinformatician managing the microarray analyses. Gene transcripts having average expression signal above background noise (>1.5) were considered significant, detected and existing, while average gene expression <1.5 was considered as "not existing". If average gene expression was <1.5 before IPC and increased to >1.5 after IPC, the gene transcript was considered to have emerged as a result of IPC and vice versa; if the signal was >1.5 before IPC and decreased to <1.5 after IPC, the gene transcript was assessed to have disappeared due to IPC. If average expression was >1.5 before and after IPC, significant up or down regulated mRNA sequences were defined as log 2 Fold Change (log 2 FC) ± 0.5.

From studies on gene expression of cells *(in vitro),* it is common set higher limits for significant average gene expression and log 2 FC. In our study we had to consider that we analyzed gene expression of EV, which has much less mRNA content compared to cells. Further, we studied gene expression of *in vivo* material, which generally contains less mRNA content compared to *in vitro* material.

DNA – next generation sequencing (NGS) (Paper IV)

The extracted DNA from EV derived from all five pigs was pooled separately before and after IPC. This was done before the DNA sequencing which was performed by National Genomics Infrastructure, in Uppsala. The bioinformatics analysis of data was achieved in collaboration with a bioinformatical consultant from the Bioinformatics Infrastructure of Life Science in Uppsala.

Bioinformatic assessment – ortholog analysis, association to cardioprotection (Paper III)

Since the functions of many porcine genes are not annotated, orthologous genes were identified from well-explored homo sapiens using the web-based tool UniProt. GeneCards, a web-based database of human genes, provided functional information

about the genes. The biological function could be determined in 70% of increased EV mRNA sequences. The web-based database PubMed was used to identify gene transcripts with association to cardioprotection and I/R or IPC.

Bioinformatic assessment – gene ontology (Papers III–IV)

In Paper III the gene ontology analysis was performed on the 2000 gene transcripts with highest expression (mean value from all pigs) before and after IPC. In Paper IV the analysis was performed on the 1000 genes with most reads in EV before IPC, and on those with most reads in EV after IPC. The amount of reads corresponds to the number of times a certain gene has been sequenced during analysis. The gene ontology analyses were performed using the functional annotation clustering in the web-accessible program DAVID Bioinformatics. Clusters having enrichment score >1.3 were regarded as significant (119).

Statistics

The data in Paper I were presented as mean ± standard error of the mean (SEM). In Paper II demographic variables were presented as mean ± standard deviation (SD) and variation for other variables as mean ± 95% confidence interval (CI). For all variables in Papers I and II, Kolmogorov-Smirnov test was applied for assessing normality, since this is a requirement for further analysis using parametric tests. The t-PA fluxes and cumulative t-PA release were evaluated by repeated measures analysis of variance (ANOVA, mixed between-within ANOVA) in Paper I and repeated measures ANOVA for cross-over design in Paper II. T-tests (unpaired t-test in Paper I, paired t-test in Paper II) were further used for single contrasts between groups. The carry-over effect was assessed by testing the difference between Group A and Group B with respect to the mean value of cumulative t-PA at 1.5 min for VPA-1 and control-1, using t-test. All tests were two-tailed and p-values <0.05 were considered significant. Statistical analysis was performed using SPSS. A power analysis was performed when designing Paper II. An approximation of 30% increase in t-PA release due to VPA, variability of 50% within-group (38), together with α value of 0.05 and power of 0.8 estimated a sample size of 22 subjects.

In Paper III gene expression log 2 FC was calculated as the mean value before IPC versus the mean value after IPC. Statistical significance was stated as p-value <0.05. The differences in gene expression before and after IPC were detected by an empirical Bayes moderated t-test, using the limma software package (120, 121). Due to problem with multiple testing, p-values were adjusted by the method of Benjamini and Hochberg (122).

In Paper III and IV, descriptive variables were presented as mean ± SD.

Results

Paper I

Coronary ischemia resulted in t-PA release throughout the post-ischemic period for both VPA treated and control animals. As an illustration, Q_{GCV} and t-PA data from one representative animal is shown in Figure 9.

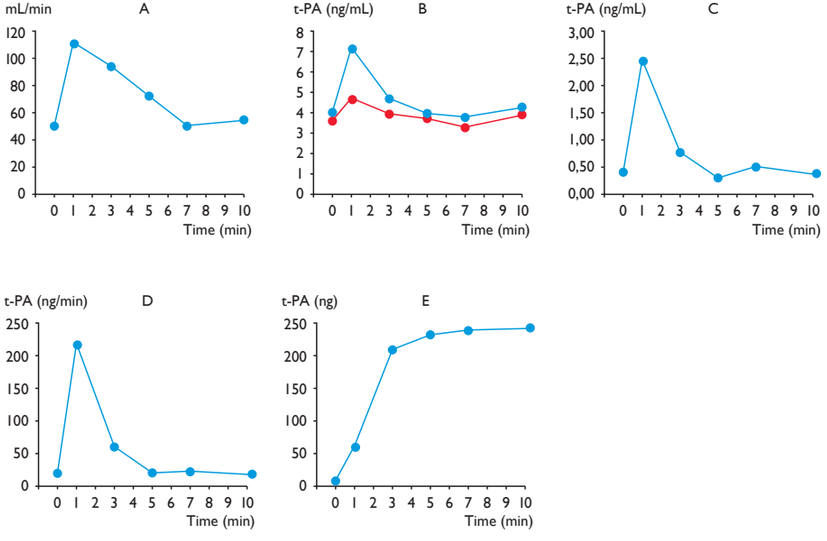

Figure 9. Demonstration of Q_{GCV}, t-PA data and t-PA calculations (data from one animal). In panel A, coronary blood flow (Q_{GCV}) is shown before ischemia (0 min) and during reperfusion. Panel B demonstrates venous (blue) and arterial (red) t-PA concentrations, which are used to calculate the veno-arterial t-PA concentration difference shown in panel C. Panel D demonstrates absolute t-PA fluxes at each time point, calculated by the product of Q_{GCV} and the veno-arterial t-PA difference (panel A and C). AUC in panel D is used to calculate cumulative t-PA release over time, shown in panel E. Abbreviations in figure: ng=nanograms, mL=millilitres, min=minutes.

Absolute coronary t-PA fluxes, at baseline and after ischemia, are shown for both groups in Figure 10. Our main finding was that the VPA treated animals showed a significantly higher cumulative coronary t-PA release compared to controls, 932 ± 173 ng versus 451 ± 78 ng (mean ± SEM), at 10 min of reperfusion. The difference was detected already at 5 min (Figure 11). No difference in static PAI-1 levels was detected between groups.

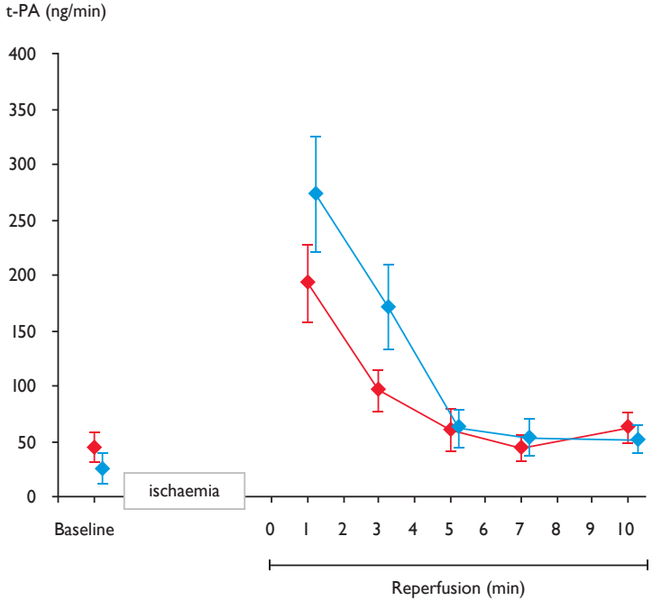

Figure 10. Coronary t-PA fluxes. Absolute t-PA fluxes are shown at baseline and during reperfusion at each point measurement, for treated animals (blue diamonds, n=12) and controls (red diamonds, n=10). Data are presented as mean ± SEM. Abbreviations in figure: ng=nanograms, min=minutes. Figure adapted from Paper I with permission from the publisher.

There was no difference between the treated and control group in Q_{GCV} or lactate production due to coronary ischemia (Paper I, Table 1 and Figure 2). Arterial t-PA (reflecting systemic t-PA levels) was not affected by coronary ischemia, and there was no significant difference in arterial t-PA levels between treated and controls (Paper I, Figure 3). Serum VPA concentration could not be detected in any of the animals.

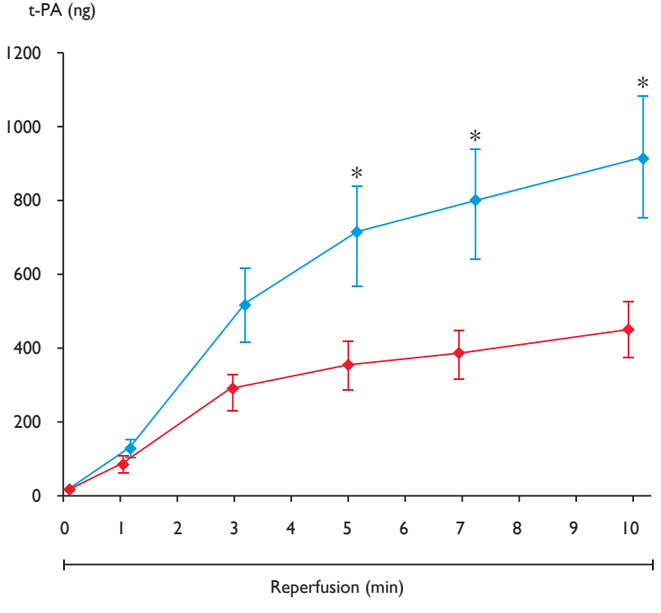

Figure 11. Cumulative coronary t-PA release. The VPA treated group (blue diamonds, n=12) showed a higher cumulative t-PA release over time than control animals (red diamonds, n=10). The cumulative t-PA release in the treatment group was larger at 5, 7 and 10 min. * = $p<0.05$ (unpaired t-test). Data are presented as mean ± SEM. Abbreviations in figure: ng=nanograms, min=minutes. Figure reproduced from Paper I with permission from the publisher.

Paper II

There was no significant difference in cumulative t-PA release during single stimulation when comparing VPA-1 and control-1 (Figure 12) or VPA-2 and control-2 (data not shown).

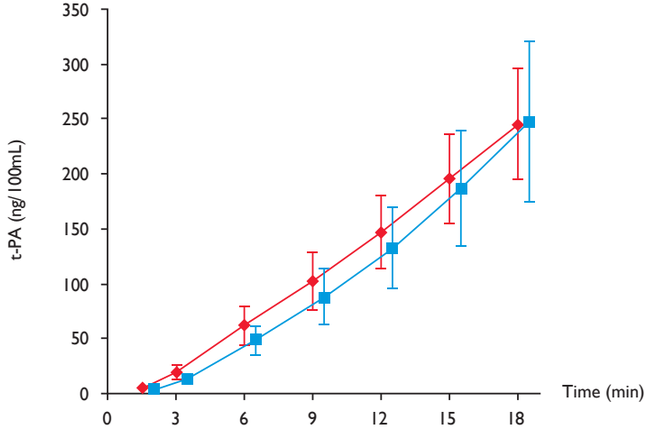

Figure 12. Cumulative t-PA release during first stimulation. There was no significant difference in cumulative t-PA release between VPA-1 (blue squares, n=15) and Control-1 (red diamonds, n=16). Data are presented as mean ± 95% CI. Abbreviations in figure: ng=nanograms, mL=millilitres, min=minutes.

The main findings were that there was an early pattern of exhaustion of cumulative t-PA release (decreased release during second stimulation) in the control group whereas exhaustion after VPA treatment was detected first at 18 min (Figure 13). In Figure 14 the degree of exhaustion was calculated for each individual (VPA-2/VPA-1 and control-2/control-1) and then, the ratios were grouped. The analysis was performed to compare exhaustion between VPA and control measurements. Valproic acid treatment demonstrated significantly less exhaustion in cumulative t-PA release from 6 to 15 minutes (Figure 14). Moreover, static plasma PAI-1 levels were decreased after VPA treatment compared to controls (18.4 ± 10.0 versus 11.0 ± 7.1 ng/ml, mean ± 95% CI, p=0.01).

We could also demonstrate a consistent pattern of the t-PA release (fluxes) over time for controls while VPA treatment led to a steady, linear increase in t-PA release (fluxes) over time (Paper II, Figure 2).

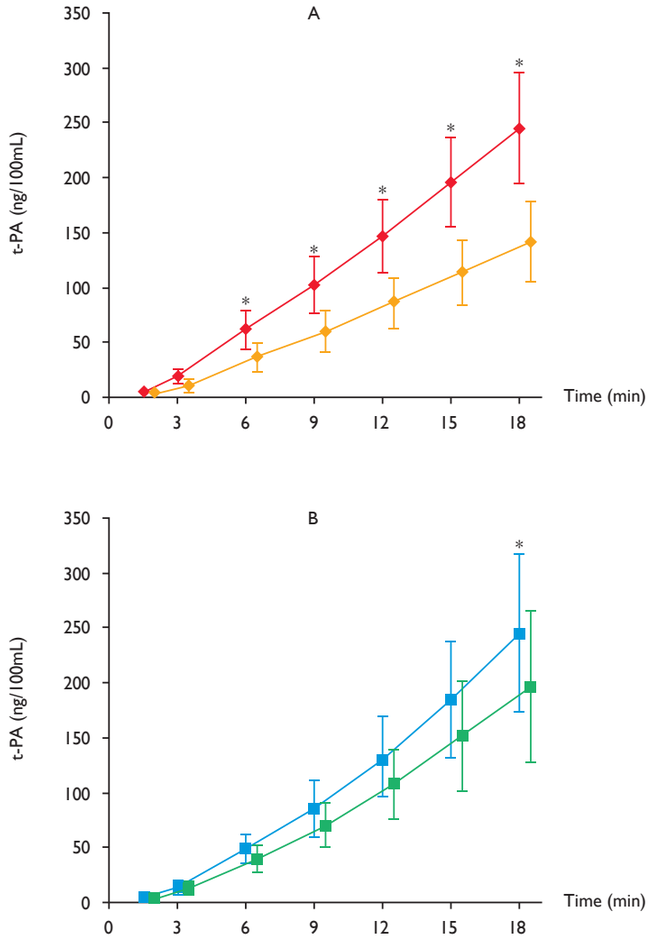

Figure 13. Exhaustion in cumulative t-PA release. Panel A demonstrates cumulative t-PA release for Control-1 (red diamonds, n=16) and Control-2 (orange diamonds, n=15), and panel B for VPA-1 (blue squares, n=15) and VPA-2 (green squares, n=15). Exhaustation (reduced t-PA release during second stimulation) is shown already at 6 min for controls and at 18 min after VPA treatment. * = p<0.05 (paired t-test). Data are presented as mean ± 95% CI. Abbreviations in figure: ng=nanograms, mL=millilitres, min=minutes. Figure reproduced from Paper II with permission from the publisher.

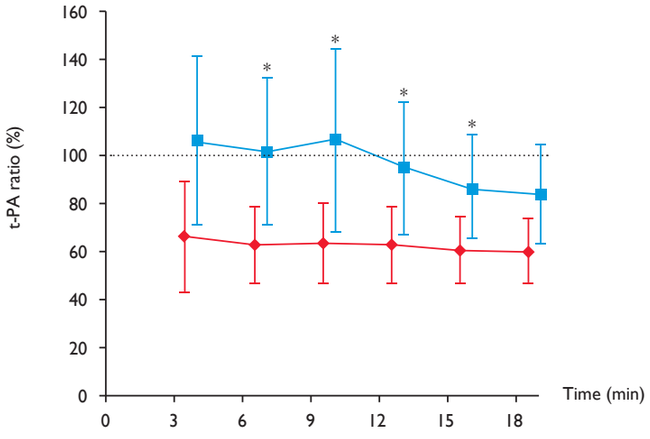

Figure 14. Comparison in exhaustion of cumulative t-PA release between VPA and controls. Exhaustion in cumulative t-PA release is compared between Controls (red diamonds, n=13, calculated as control-2/control-1) and VPA (blue squares, n=13, calculated as VPA-2/VPA-1). There is a steady pattern of exhaustion from start in controls whereas with VPA the exhaustion in t-PA release starts first after 9 min. The exhaustion is less with VPA treatment from 6 min until 15 min. * = $p<0.05$ (paired t-test). Data are presented as mean ± 95% CI. Abbreviations in figure: min=minutes. Figure reproduced from Paper II with permission from the publisher.

There was no difference in t-PA release between subjects receiving VPA treatment first, and those given treatment after control measurements, which means that no period-effect was detected (p=0.98 and 0.99 for control-1 and VPA-1, data not shown). No carry-over effect could be shown (p=0.82). The ISP stimulation resulted in a rapid approximate 300% increase in FBF that was sustained throughout the infusion period, with no difference between first and second stimulation sequence (data not shown).

Valproic acid was detected in serum of all subjects after treatment with a mean concentration within therapeutic levels for anticonvulsive treatment (one individual was just below the lower limit). The VPA treatment was associated with a small but significant decrease in platelet count, fibrinogen, hs-CRP and a significant increase in PT-INR, although all mean values were within the normal range (Paper II, Table 2). TEG analysis did not show any difference in clot lysis index (a percentage of clot stability in relation to its maximal strength after 30, 45 and 60 min) after VPA treatment compared to control (data not shown).

Paper III

Gene ontology analysis demonstrated an enrichment of EV mRNA coding for proteins associated with regulation of transcription, translation, extracellular matrix, morphogenic development and feeding behavior.

Microarray assessment detected 11678 different mRNA sequences in EV before and/or after IPC. A total of 1103 (9.5% of all detected) gene transcripts were significantly increased or decreased after IPC. Here among, 638 mRNA sequences increased or emerged after IPC. Several of these sequences are known to be associated to cardioprotection during I/R or IPC and five sequences are especially strong associated. These sequences were coding for the transcription factors: signal transducer and activator of transcription-3 (STAT3) and T-cell transcription factors/lymphoid-enhancer binding factor (TCF/LEF), the enzymes: cyclooxygenase-2 (COX-2) and glycogen synthase kinase 3 beta (GSK-3β) and the receptor ryanodine receptor type 2 (RyR-2) (123–128).

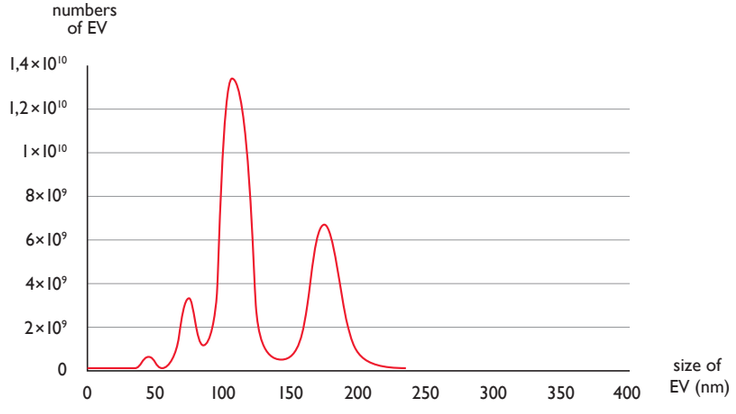

Figure 15. Nanoparticle tracking analysis result from one animal during reperfusion. The figure shows an example of EV populations of different sizes, ranging from approximately 50 to 200 nanometers (nm). The x-axis denotes EV size and the y-axis denotes number of EV.

There was a large variation in EV size between animals, as well as before and after IPC. No pattern of change after IPC was identified. The EV size ranged from 30 to 350 nm (nanometer), in which most EV were between 75 and 200 nm. There was also a large variation in EV amount between animals, as well as before and after IPC. The mean EV plasma concentration at baseline was (2.9 ± 2.7) • 10^{11}/mL, and after IPC (1.6 ± 1.9) • 10^{11}/mL. Figure 15 illustrates an example of a NTA result from one animal during reperfusion. Western blot, EM and NTA together evaluated the EV preparation. CD81 was detected in EV samples but not in the porcine cell suspension and GRP78 in cell suspension but not in EV samples. This indicates EV presence and absence of contaminating cells or apoptotic bodies in the EV isolation. The EM image illustrates a typical EV of similar size as detected by NTA (Figure 16).

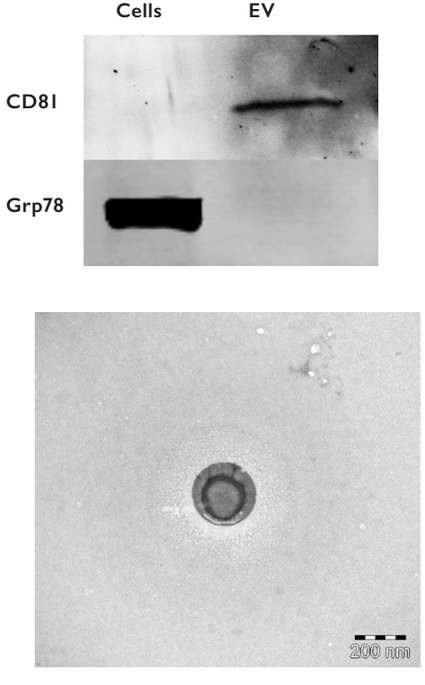

Figure 16. Electron microscopy image of isolated EV (top) and western blot results (bottom). The figures demonstrate a quality aspect of the EV preparation. Here, showing isolated particles as EV and absence of contaminating apoptotic bodies or cells. Abbreviations in figure: nm=nanometers. Figure adapted from Paper III.

Paper IV

Genomic DNA was detected in EV both before and after IPC. The DNA sequences represented all chromosomes of the porcine genome, including porcine mitochondrial DNA.

The DNA content in plasma-derived EV changed after IPC. When aligning all DNA sequences to respective chromosomes, an average increase of 16% of unique DNA sequences could be observed (15.9 ± 3.6%, mean ± SD).

The 1000 genes with most reads in EV isolated before IPC were compared to the 1000 genes with most reads in EV after IPC. A total of 494 genes were detected in EV both before and after IPC. In total, 506 genes in EV before IPC were not found among the genes in EV after IPC, and vice versa 506 genes in EV after IPC were unique compared to the genes in EV before IPC.

Gene ontology analysis of the 506 unique genes in EV before IPC showed an significant enrichment of DNA coding for ion channels, membrane proteins, mitochondrial proteins, proteins involved in lipid metabolism and posttranslational modifications. In contrast, the analysis could not detect an enrichment among the 506 unique genes in EV isolated after IPC.

Evaluation of the EV isolation is described in the result section of Paper III.

Discussion

The aim of this thesis was to explore aspects of epigenetic regulation affecting endogenous protective mechanisms against myocardial ischemia. More specifically, Papers I and II focused on endogenous tissue protection in the event of thrombotic threats by exploring epigenetic regulation of t-PA and stimulation of the fibrinolytic system *in vivo*. Papers III and IV focused on endogenous cardioprotection during IPC by studying dynamics in genetic content in EV (as potential epigenetic modifiers) collected from coronary venous blood in an *in vivo* myocardial IPC setting.

HDACi treatment augments the fibrinolytic system *in vivo*

We conclude that HDACi treatment, using VPA, enhances and improves t-PA release capacity and the fibrinolytic system *in vivo* in a large animal model with healthy endothelium as well as in a clinical cohort with atherosclerotic disease. This is the first demonstration of this effect *in vivo*.

Influence of t-PA release capacity

In Paper I it was demonstrated that the epigenetic pharmacological intervention of HDACi, by oral VPA treatment, improved the endogenous fibrinolytic system by increasing cumulative coronary t-PA release in a porcine myocardial ischemia model. Despite the relatively low VPA dose, it enhanced t-PA release capacity in this model. One could speculate that if there is a dose-relation for this effect, a higher dose might produce an even greater increase in t-PA release. The plasma VPA concentrations in our study were below detection levels 12–18 hours after last administration of VPA, showing rapid clearance. Oral VPA has lower bioavailability and a markedly lower half-life in most species compared to man, although this has not been studied in pigs (129). In our study, laboratory assistants confirmed that the animals swallowed the VPA pills as dosed, since they were delivered in small amounts of sponge cake (laboratory assistants choice) that the animals consumed with great enthusiasm.

In Paper II, we could not demonstrate the same difference in cumulative t-PA release during single stimulation between VPA and control measurements, as in Paper I. There can be more than one explanation why the result from the animal study was not reproduced in this clinical trial. Humans and pigs can be presumed to have different pharmacokinetics and pharmacodynamics related to VPA. In addition, the two studies employed different t-PA release stimuli with various potencies (myocardial ischemia and ISP) in different vascular beds (cardiac and forearm) with diverse t-PA release capacity, in healthy and atherosclerotic endothelium (22, 31, 51).

Despite no differences in cumulative t-PA release for single stimulated sequences, the findings in Paper II illustrated other aspects of enhanced fibrinolytic response related to VPA treatment. Valproic acid augmented cumulative t-PA release capacity during repeated stimulation sequences; VPA treatment resulted in better preservation of t-PA release during second stimulation compared to controls. This effect may have clinical relevance in recurring, prolonged or massive thrombotic events. One interpretation might be that reduced exhaustion/preserved t-PA release during repeated stimulation can be based on increased t-PA synthesis and larger intracellular depots of t-PA due to HDACi increasing t-PA gene expression. Paper I did only examine single stimulation of t-PA release, and in reflection, a repeated stimulation protocol could have been possible to perform with the coronary ligation model as well. The idea of repeated stimulation sequences, to test endurance in t-PA release, emerged during discussion of the preliminary results from Paper I and was incorporated into the study protocol of Paper II.

Influence of PAI-1

In Paper II it was also demonstrated that VPA treatment in human atherosclerotic subjects additionally favored the fibrinolytic system by causing a decrease in plasma PAI-1 levels. This effect has previously been detected in an *in vitro* study and also recently in a human model (130, 131). The main source of plasma PAI-1 is from platelets (132, 133). Our data showed a simultaneous decrease in platelet counts, although it was not large enough to be proportional to the decrease in PAI-1 levels. Therefore, this cannot explain the pro-fibrinolytic state. Unpublished data from a mouse model have shown an inverse dose-response relationship between high concentrations of VPA and decreased concentrations of PAI-1 (personal communication, Niklas Bergh). This might explain why PAI-1 was not decreased due to VPA treatment in the porcine model, where the VPA concentrations were below detection levels. Further studies are needed to elucidate the mechanisms behind the down regulatory effect of VPA on PAI-1 levels.

Treatment perspectives concerning HDACi and stimulated fibrinolysis

The influence of VPA on the fibrinolytic system *in vivo* is supported in a large retrospective study of antiepileptic treatment with VPA where a 40% risk reduction of myocardial infarction in epilepsy patients with VPA treatment (compared to age- and gender matched controls from population) was shown (134, 135). A similar association was recently published in a nested case-control study (136). The findings might partly be related to enhanced t-PA release capacity and/or decreased PAI-1 levels with HDACi treatment, as was detected in Papers I and II.

Valproic acid treatment did not derange plasma coagulation status and TEG results were not affected (Paper II). Additionally, VPA did not influence either constitutive t-PA release (baseline t-PA fluxes) or systemic (arterial) t-PA levels during stimulation. Hence, t-PA was only released locally and when it was needed. This confirms the hypothesis that HDACi increases t-PA gene expression and synthesis. The t-PA is further stored in intracellular depots and is secreted when the endothelium is stimulated (where and when it is required). This is clinically important, both for the time aspect of fibrinolysis as well as in context of potential adverse effects (unwanted bleeding in organs not affected by thrombosis). In a clinical situation where bleeding is associated with an unhealthy large fibrinolytic response, it is pharmacologically possible to inhibit t-PA and conversion of plasminogen to plasmin by use of anti-fibrinolytic agents such as tranexamic acid. These aspects make HDACi treatment promising as a new and potentially better approach for preventing thrombosis formation while not causing deleterious bleeding side-effects.

Even though epigenetic agents enhancing t-PA release capacity seem attractive for CVD treatment, the pharmacological principle is also potentially associated with clinical relevant adverse effects. First, t-PA is also produced by other cell types and has physiological and pathological roles outside the vascular system, such as in the central nervous system (CNS) and peritoneum (23). Although, unpublished data recently showed that CNS cells are much more resistant to HDACi concerning t-PA synthesis compared to endothelial cells (personal communication, Niklas Bergh). Secondly, when inhibiting HDAC, the expression of genes other than t-PA may also be affected. *In vitro* studies have indicated that approximately 2% of human genes are affected by VPA or other agents modulating histone acetylation status (67, 137). Furthermore, HDAC also have non-histone proteins substrates such as transcription factors (138). Currently, there are some HDACi approved for clinical practice within cancer treatment, and many are being investigated in different phases of clinical trials. With doses used for oncological treatment, nausea, vomiting and fatigue are commonly observed side-effects, together with transient thrombocytopenia, neutropenia and anemia. Further, ECG changes, liver toxicity, confusion and somnolence are seen (13). However, the increased t-PA production *in vitro* has appeared at much lower doses of different HDACi compared to doses required for cancer treatment (61). In the *in vivo* setting of Paper II, side-effects were not common. One subject suffered from mild gastrointestinal side-effects, and there was a significant decrease in the average platelets count (although still within normal range). Thus, when HDACi may be used for an antithrombotic indication, plasma concentrations are low enough to expect reduced risk of side-effects. It should also be noted that normal cells, in contrast to tumor cells, are relatively resistant to the HDACi antitumor effects (such as induced cell cycle arrest, activation of apoptotic pathways and induced cell death) (138).

Methodological considerations in Papers I and II

Animals (Paper I)

Previous *in vitro* findings showing that t-PA is under epigenetic control by HDACi and VPA provided the basis for the hypothesis in Paper I (67). An animal model was chosen to explore this *in vivo*. Pigs share many anatomical and physiological characteristics with humans, especially regarding the cardiovascular system. This includes heart-to-body weight ratio, coronary artery anatomy and (unlike other laboratory animals) low extent of collateral circulation (139). These qualities make the pig an ideal animal for studies on the heart and coronary circulation. Furthermore, creating myocardial ischemia and then performing blood sampling across the coronary vascular bed would not have been applicable in a small animal model. There was no power analysis made during planning of this experiment since estimation of expected VPA treatment effect was impossible to assess. Previous results from *in vitro* studies on VPA and t-PA would not be appropriate to use.

Study design and protocol (Paper I)

Several physiological and pharmacological factors stimulate t-PA release. Two years of methodological work (using 34 pigs) was performed, in order to get a robust protocol for Paper I. Several t-PA stimuli factors were tested in the pilot trials, such as myocardial ischemia by LAD occlusion (open-chest model) and intracoronary administration of substance P, bradykinin and ISP (closed-chest model). The coronary ischemic stimulus (transient and reversible LAD occlusion) is a well-practiced method used in our laboratory and it was finally chosen as the best model for this study (36). Sternotomy is a known t-PA release stimulus (140) but since all animals were treated the same way and the protocol included one hour of rest after surgical preparation, we did not consider this as a disturbing factor. The middle portion of the LAD was occluded. Pigs have a high susceptibility to develop malignant arrhythmias during I/R, where the size of the ischemic myocardium is a major risk determinant. From previous studies we knew that ten minutes of coronary occlusion would stimulate t-PA release without high risk of arrhythmias and sudden death (36).

VPA dose-regime (Paper I)

There was no published pharmacokinetic data on oral administrated VPA in pigs when we were planning the study. It was decided to evaluate the effects of a single dose of VPA on t-PA release in this first in vivo trial, without assessing a dose-response relationship. The VPA dose was selected based on dosing in humans, aiming to reach plasma VPA concentrations similar to therapeutic levels in epileptic patients and avoid side-effects. Similar VPA concentrations had been used in the cell culture media of

an in vitro study (although it is not appropriate to transfer laboratory conditions to in vivo trials) (67). A one-week treatment of VPA was chosen as a compromise between a goal of treatment over many days and the practical limit of animal housing in our large animal facility. The animals in the intervention group spent more days in the animal research facility pens, though these were similar in nature to their usual pens in which they were raised. The animals were siblings, and accustomed to each other's company (no fighting or signs of stress were observed).

Subjects (Paper II)

The study was performed in a homogeneous cohort to limit confounding factors and the numbers of individuals needed. Women were excluded to eliminate endocrinological effects influencing t-PA release (23, 141). An earlier similar trial on healthy subjects, with two weeks of VPA treatment and single sequence stimulation, did not demonstrate an enhanced t-PA effect (131). One interpretation of those findings may be that it can be difficult to generate supra-physiological t-PA release in healthy endothelium by using VPA treatment. We hypothesized that VPA could increase t-PA production and secretion in subjects with deficient t-PA release capacity and therefore, we aimed to study an atherosclerotic cohort. Subjects that had been treated for a myocardial infarction and were suffering from left main stenosis or 2- or 3-vessel disease were chosen. In order to avoid dual antiplatelet therapy (aspirin and a second antiplatelet agent is recommended first year after the infarction (142)) subjects that had been diagnosed and treated for a myocardial infarction more than one year ago were chosen.

The power analysis suggested that 22 subjects would be needed. Initially, 23 subjects were included, but due to dropouts only 16 subjects completed the study. However, since the power analysis was based on a modest estimated effect size (30%) and a large variability (50%), we decided to analyze data. When the analysis showed a treatment effect of VPA we chose not to re-open the study to include more subjects.

Study design and protocol (Paper II)

The subjects had specific food and behavioral restrictions before and on test days (108–112). During the experiment subjects were resting in a reclining position in a quiet room to further minimize t-PA disturbing factors (143).

The study aimed to evaluate single stimulated t-PA release due to VPA treatment and, in addition, repeated stimulated t-PA release capacity as a marker of cellular t-PA depots. Since the participants were fasted and placed in a chair not able to move, the study was limited to two t-PA stimuli sequences as this procedure took about five

hours. The t-PA release capacity was assessed using the forearm vascular bed, which is a vascular bed not closely associated with atherosclerosis or thrombotic events. The ideal model would have been to study the coronary vascular bed, but due to risks and the invasive nature of such procedure, it was not ethically reasonable in this first clinical trial. Stimulated t-PA release in the perfused forearm model has been considered a reliable method and reasonable surrogate vascular bed to study t-PA release patterns in humans (22). Substance P was an initial alternative to stimulate t-PA release, because of its potency and published experience from others, but it was not commercially available when our study was performed (131, 144). The dose-regime of the pharmacological ISP stimuli was based on a previous report (38).

A cross-over design protocol was used in the study. This allowed each subject to serve as their own control and avoid inter-individual variability when comparing treated and control stimuli sequences. To eliminate effects of stress and anxiety on stimulated t-PA release, participants were randomly allocated to have the VPA treatment sequences either before or after the control sequences. From our findings, there was no difference in t-PA release if subjects received VPA treatment before or after control measurements (no period-effect) and therefore data from control and VPA measurements were grouped before statistical analysis (Figure 6).

VPA dose-regime (Paper II)

Cumulative coronary t-PA release was enhanced in the porcine myocardial ischemia model following one week of VPA treatment (500 mg, twice daily). The VPA dose (Ergenyl Retard 500 mg, twice daily) in Paper II was chosen in accordance to this finding, as well as extrapolated from previous in vitro data (67). In order to minimize side effects, we chose a VPA dose aiming to reach the lower range of plasma concentration used in anticonvulsive therapy. The study protocol of Paper II differed from that in Paper I (clinical subjects, forearm vascular bed, atherosclerotic disease and pharmacological stimulation). Four weeks of VPA treatment was considered reasonable based on pharmacokinetic knowledge of VPA in humans (about three days of oral drug administration is required to reach a predictable steady state) together with experience from another study by our group (131). Since acetylation status of histones is dynamic and changes rapidly (hours or days) when HDACi is not present, the washout period (at least four weeks after VPA treatment, before control measurement) was considered long enough to presume no carry-over effect (145, 146).

Limitations (Papers I and II)

Both trials were relatively small with limited numbers of animals (n=22) and clinical subjects (n=16). The studies were not blinded, placebo-controlled (from pecuniary as-

pects) or randomized. They were not designed to evaluate a dose-response relationship between VPA treatment and t-PA release capacity. The degree of histone acetylation status in endothelial cells (or other cells) was not measured. In retrospect, this would have been methodologically confirmatory for the presence of HDACi effect, since reports in humans have shown an individual variation in histone acetylation for the same VPA dose (64). Also, histone acetylation measurements could have confirmed that the degree of acetylation in control measurements were homogeneous (Paper II). The effects of VPA on histone acetylation status have previously been confirmed in several in vitro studies using different cell types, and in vivo, in both animal and human experiments (62–67). The amount of endothelial cellular t-PA depots was not evaluated in our studies. Coronary vessel biopsies from animals could have been obtained and analyzed, but would also have required extensive methodological development. In humans, forearm endothelial biopsies would have been needed and therefore it was not considered.

Neither the animal nor the clinical model evaluated t-PA release capacity due to stimulation by a thrombotic event, which is more clinically relevant than evaluating t-PA release stimulated by myocardial ischemia and ISP infusion. Models of coronary artery thrombosis induced by copper coil have been described (147), but could not have been applicable in Paper I since coronary venous t-PA sampling during a thrombotic occlusion would have been problematic. Primary endpoints of both studies were quantification of t-PA release patterns. There was no functional assessment evaluating whether the enhanced fibrinolytic system truly improves degradation of an intravascular thrombus or decreases the area of myocardial infarction. The ultimate test of an improvement of the fibrinolytic system is to verify that an increase t-PA release capacity and suppressed PAI-1 levels result in less thrombus formation. However, few models are suitable for this type of experiment. Many of the traditional ex vivo models, which have been used for evaluating anticoagulation agents, are not necessarily helpful since the fibrinolytic system is dependent of an intact endothelial cell layer. To our knowledge there is one acute pulmonary embolus model that can be suitable for this purpose in coming studies (148).

Myocardial IPC and extracellular vesicle genetic content

We could identify the existence of DNA content in plasma-derived EV from an in vivo porcine model. The DNA fragments detected in EV represented all chromosomes of the porcine genome, including mitochondrial DNA. This is a novel finding, which may have further implication in coming EV studies. During EV isolation, the EV pellets were treated with nuclease to remove DNA located exterior to vesicles. The identified DNA fragments were specific for the porcine genome and could not be a result of contamination.

Gene ontology analyses of EV DNA and mRNA showed enrichments of genetic material coding for proteins associated with protein synthesis in general. This is consistent with the enrichment of mRNA coding for ribosomes that has been identified in EV from cultured cardiomyocytes (87). One may speculate that one purpose of EV content is to support target cells in protein synthesis.

The main findings in Papers III and IV demonstrate that myocardial IPC influences the composition of DNA and mRNA in EV isolated from coronary venous blood. The DNA content in plasma-derived EV was different after the in vivo myocardial IPC provocation in several aspects. There was a nearly 16% increase in unique DNA sequences for each chromosome found in EV isolated after IPC. Moreover, the genes with most reads in EV before IPC were not the same and they were coding for different groups of proteins compared to genes with most reads in EV after IPC. From these findings we cannot explain if and how the dynamics in EV DNA content is related to cardioprotection and IPC.

The mRNA sequences in EV secreted from preconditioned cells were different from the mRNA content in EV released by non-preconditioned cells. Among EV gene transcripts that emerged or increased after IPC, several were coding for proteins associated to cardioprotection. Our findings support the idea of a biological dynamics of EV DNA and mRNA content, as well as the importance of the external milieu on parental cells influencing the genetic content of secreted EV. Furthermore, this may support the hypothesis that parental cells may have the capacity to design tailor-made EV with specific genetic content, when they are exposed to IPC. These EV may be capable of inducing epigenetic modulations in recipient cells.

Myocardial IPC increases EV mRNA content associated to cardioprotection

Approximately 10% of 11678 gene transcripts detected in EV were significantly up or down regulated after myocardial IPC. Among these, nearly 60% increased or emerged after IPC. Several of these sequences were coding for proteins known to be associated to cardioprotection and some with strong association to myocardial IPC; such as *STAT3, TCF/LEF, COX-2, GSK-3β and RyR-2*.

The transcription factors STAT3 and TCF/LEF have both been associated with cardioprotection by anti-apoptotic effects and improved mitochondrial function (123, 124, 128). In addition, they both initiate expression of *COX-2,* an enzyme known to be increased by IPC and mediate cardioprotection (125, 149, 150). The COX-2 enzyme and its specific cardioprotective prostanoid metabolites (PGE_2 and PGI_2) have been shown to increase at 24 hours after IPC, while *COX-2* mRNA levels rise already at first hour after intervention (151, 152). It is possible that gene expression of *STAT3*

and *TCF/LEF* is induced in EV, first in order to obtain direct cardioprotective effects and secondly to achieve COX-2 transcription in target cells. Since mRNA sequences of *COX-2* increased in EV after IPC, this might represent a potential alternative pathway to obtain COX-2 mediated cardioprotection in recipient cells. It has been suggested that HIF-1α (hypoxia-inducible factor 1α) is necessary for cardioprotection in IPC. The transcription factor is induced during hypoxic conditions and initiates expression of various cardioprotective genes such as *COX-2* (153), consistent with the present result.

Gene transcripts of *GSK-3β* were also induced in EV after myocardial IPC. The enzyme GSK-3β, in phosphorylated state, has been strongly associated with cardioprotection during IPC by inhibiting the opening of mitochondrial permeability transition pores which otherwise leads to cell necrosis (126, 154). During ischemia, the myocardial Ca^{2+} channel receptor RyR2 is impaired, resulting in cytosolic Ca^{2+} overload, which is an important pathophysiological mechanism involved in reperfusion injury (155). It has been demonstrated that myocardial IPC modify RyR2 and improve cellular Ca^{2+} homeostasis (127, 156, 157). In addition, pharmacological agents able to prevent RyR2 dysfunction have been shown to be cardioprotective (127, 155). In our study, the mRNA content of RyR2 increased in EV after IPC, although, in this context, we cannot explain how this may mediate cardioprotection.

Our findings support the idea that myocardial IPC may generate EV which communicate cardioprotection by transporting and delivering mRNA sequences, in addition to miRNA fragments as recently described (101, 102).

Myocardial IPC and extracellular vesicle size and amount

In this initial in vivo EV study, we aimed to explore signs of difference in EV size and number after IPC, since such findings could indicate an association and be useful information in generating hypotheses for future research. There was a large variation in EV size and numbers between animals, without any identifiable signs of change after IPC. The NTA showed a pattern of EV subpopulations of different sizes for each animal, before and after IPC (Figure 15). This result may be explained by the fact that the isolated plasma-derived EV from our in vivo studies presumably originate from different cell types, and different cells may generate EV of various sizes (158). Moreover, there are different types of vesicles in plasma, including exosomes and MV of various sizes (15). Vesicle aggregation occurring during ultracentrifugation could also explain that there are vesicles of many sizes (although of less amount) as seen in Figure 15. The EV concentrations in our samples were within the same range as other groups have presented (15, 16).

Treatment perspectives concerning extracellular vesicles

Extracellular vesicles are natural, biological particles with many qualities making them attractive for treatment purposes. They have therapeutic potential and offer advantages as being naturally occurring, cell-free and with limited immunogenicity. Their cargo is protected from degradation in the circulation (72) and they are labeled by membrane proteins which might help them to attach to specific target cells and further deliver their cargo directly into the cytoplasm. Further, they provide a functional aspect by mediating epigenetic modulations to recipient cells when transferring DNA and RNA (17, 19, 21). Also, EV may be stored (frozen) without potentially toxic preservatives for several months without loss in function (72).

Extracellular vesicles were recently suggested to have the potential to mediate cardioprotection in ischemic or infarcted hearts as well as in IPC, although the mechanism is not known (89, 90, 92, 93, 100–102). Myocardial IPC is an effective cardioprotective intervention, expressed as increased tolerance for subsequent myocardial ischemia. Therefore, it is important to further understand the underlying mechanisms providing this cellular defense. Such knowledge may provide basis for generating pharmacological therapeutics, which may confer the same degree of cardiac protection as IPC. One way might be to use EV from cell cultures that are created to produce target-designed EV loaded with specific cardioprotective signals (synthetic drugs, proteins, RNA or DNA). Also, EV generated from IPC cells may be isolated, stored and later given to patients with threatening vital organ injuries, such as myocardial infarction.

The results of Paper III and IV support the idea that EV may hold promise in this therapeutic field and support further research. Our studies are initial steps in exploring a potential future era of therapies based on EV carrying specific protective RNA and DNA sequences addressed to cells in danger, although much further research is needed before clinical application may be considered.

Methodological considerations in Papers III and IV

General considerations

Most studies that have suggested EV as endogenous agents delivering cardioprotective signals have been performed on in vitro and ex vivo derived EV (89, 90, 92, 93, 100, 101). Extracellular vesicles released from in vitro cell lines or ex vivo settings could be quite different compared to EV released in vivo. Previous reports have also been confined to the protective effects of EV miRNA (89, 90, 92, 93, 101, 102). Paper III and IV were designed to describe changes in EV content of DNA and mRNA related to myocardial IPC in vivo. The studies are initial investigations and they were not designed to confirm mechanistic and functional effects of the changes in EV content.

Studies that concern global gene expression and DNA sequencing are generally not used to find connections between an intervention and results. The analyses are very expensive and produce large amount of complex data. Most commonly, the purpose of these analyses is to generate novel hypothesis for further studies. Therefore, power analyses are not appropriate in this context. The descriptive data and associations generated by small sample size hypothesis driven microarray or sequencing experiments need to be validated in other settings such as larger or functional studies in order to draw conclusions of wider perspective regarding the population.

In Paper IV we also aimed to identify the existence of DNA in EV derived from porcine plasma. Even though previous studies have identified DNA in EV (18, 19, 73, 81), there is no general agreement within the EV research field concerning whether DNA actually does exist in EV. One possible reason for this lack of agreement is that the methods for isolating, purifying and describing EV has evolved very rapidly, and there has been concern about confirmation of different types of EV and whether their content is altered, from their natural state in vivo, during preparation. Previous studies have demonstrated the existence of DNA in EV from human and rodents (73, 81, 86), but not in pigs. As the pig is a relevant model with many similarities to humans this is important knowledge that may provide basis for coming cardiovascular in vivo EV studies.

Animals

The hypotheses for Papers III and IV were based on in vitro discoveries showing that the environment on parental cells influences the genetic content of EV and that EV may induce epigenetic changes in recipient cells by transferring functional sequences of RNA and DNA (17–19, 75, 87). Also, EV have been proposed to mediate cardioprotection in IPC and RIPC (100–102). As previously described the pig is the ideal animal for this type of cardiovascular study. Moreover, it was essential to sample large amounts of blood before and after IPC (about 200 mL) in order to have enough EV genetic material to perform gene expression microarray and DNA sequencing analysis. The pigs are big enough to survive the required blood sampling, in contrast to rodents. Our study included five animals, which was considered reasonable for these initial studies of EV and genetic content.

Study design and protocol

The chosen porcine myocardial IPC model has been evaluated and applied for many years in our group (107, 156). There was no published knowledge to guide the timing for EV sampling (immediately after IPC, one hour or several hours later) and changes over time in EV production, content and release in response to an in vivo ischemic provocation were not known. Myocardial IPC is recognized to cause an immediate, within minutes, protection of the myocardium lasting for a few hours, followed by a delayed phase reappearing after 24 hours (95). Others have shown that coronary perfusate containing EV, collected between first and last IPC reperfusion phase, reduced myocardial infarct size when given before ischemia in an ex vivo rat model (100). Thus, a rapid change in EV content could be possible. Although, the potential protective signals in EV may generate the immediate as well as the delayed phase of cardioprotection after IPC. In our systematic approach, we aimed to start sampling early after IPC. Due to the dimensions of GcV and the CS catheter, it was impossible to rapidly draw large amounts of blood. Consequently, blood was collected more or less continuously during 20 minutes in order to collect as large blood volume as possible.

Extracellular vesicle isolation

It is challenging to purify and isolate EV and there are different possible methods to choose from; such as ultracentrifugation (with or without density gradient-based isolation), filtration, immunoisolation and commercial easy-to-use precipitations solutions (115–117). Currently, there is no consensus on a "gold-standard" or optimal method to be uniformly used to isolate and purify EV. Each has advantages and disadvantages concerning specificity, risk of impurity and EV yield (70, 116, 117). Despite good laboratory practice, no method guarantees a completely pure EV preparation without co-isolation of contaminating factors. The scientific field is new, techniques are rapidly developing, and there is need for standardization. Recently, the International Society for Extracellular Vesicles (ISEV) proposed guidelines for minimal characterization of isolated EV, although these were published after the experimental phase of Papers III and IV was completed (70). In our studies differential ultracentrifugation (with sucrose density gradient-based isolation) was applied for EV isolation. This is the most accepted and commonly used method, and results in a very pure preparation (116). However, the method provides a relatively low EV yield and it has been suggested that vesicle aggregation occur during the ultracentrifugation (158). It is also speculated that ultracentrifugation may compress vesicles letting membrane proteins from one vesicle being pushed into membranes of others. However, this was not addressed within the scope of our studies.

We evaluated the EV preparations by EM, NTA and western blot. The western blot analysis was restricted to the few commercially available antibodies for porcine antigens. In this aspect the ISEV guidelines was not completely followed.

Limitations (Papers III and IV)

Our studies were designed to describe the impact of myocardial IPC on EV content of mRNA and DNA, though not including a functional aspect in recipient cells or organs. Previous in vitro studies have shown how EV mRNA may change the phenotype of recipient cells. A recent publication has demonstrated that EV DNA, as well, resulted in increased transcription and production of mRNA and proteins in recipient cells in vitro and in vivo. We may speculate that induced EV mRNA sequences (Paper III) also are capable of changing RNA and protein syntheses of recipient cells (17–19, 21, 75).

The blood sampling was localized to the venous drainage of the myocardial ischemic area, although, obviously still mixed with blood from the whole circulation. In this way, isolated EV represent origin from other cell types and locations in the body. Currently, there is no marker or method to distinguish or isolate EV of specific cardiomyocyte origin. We reason that it is most probable that EV containing gene transcripts that changed after IPC were originating from the heart, from cells exposed to the IPC intervention. Whether these EV were derived from cardiomyocytes, smooth muscle cells, fibroblasts, endothelial cells or cardiac progenitor cells is not known. It was recently shown that coronary perfusate containing EV (without further details about EV cell origin) collected ex vivo of IPC hearts, attenuated infarct size in ischemic hearts to a similar extent as cardioprotection afforded by IPC (100).

Concerning assessment of microarray data and DNA sequencing, our analysis was guided by availability of the known porcine genome and gene functions, which are less understood than those for the human or murine genomes. Further, there are limits concerning commercially available antibodies for porcine antigens for additional analysis. Despite this the porcine model is very suitable compared to rodent as far as harvesting lots of EV from the coronary circulation and similar heart physiology compared to humans.

Conclusions

- Histone deacetylase inhibition, by VPA treatment, increases cumulative coronary t-PA release in an in vivo porcine myocardial ischemia model.
- Histone deacetylase inhibition, by VPA treatment, increases cumulative t-PA release during repeated pharmacological adrenergic stimulation but not during single stimulation in atherosclerotic subjects.
- VPA treatment decreases static plasma levels of PAI-1 in atherosclerotic subjects.
- Myocardial IPC leads to changes in gene transcripts in EV isolated from coronary venous blood immediately after intervention, including increases in mRNA sequences which have strong association to cardioprotection.
- Porcine plasma-derived EV contain DNA fragments representing all chromosomes of the porcine genome, including mitochondrial DNA. Myocardial IPC intervention changes DNA content in EV harvested from coronary venous effluent.

Future perspectives

HDACi and stimulated fibrinolysis

The findings in Papers I and II indicate that epigenetic intervention by HDACi has promising effects by improving the endogenous fibrinolytic capacity and thus may have future utility in prophylactic treatment for CVD in patients with increased thromboembolic risk. These are still preliminary findings, and this new pharmacological intervention needs to be confirmed in future studies. The functional impact of HDACi treatment on thrombus formation needs to be explored, as well as further assessment of HDACi efficacy concerning clinical relevant endpoints such as reduced risk of myocardial infarction and decreased mortality. Studies are needed concerning optimal dose regimes, developing selective, ideal HDACi to reduce risk of side effects and biomarkers to easily monitor HDACi effect.

Extracellular vesicles

Our findings in Paper III and IV, together with results by others, suggest that one may assume that parental cells, when exposed to threatening external stimuli (such as ischemia), provide specific genetic signals in EV that recipient cells may convert to proteins protecting them from damage. The results further indicate an association between myocardial IPC and EV, and that there is reason to support future research in this area.

Extracellular vesicles from in vivo IPC need to be further functional assessed. Better understanding of specific markers of EV cell origin is also a relevant future study area. Further, knowledge of EV selectively finding target cells together with characteristics and dynamics of EV membrane proteins requires exploration. More knowledge is needed about the influence of external stimuli on quantitative and qualitative aspects of EV release over time.

Despite the fact that basic fundamental preclinical studies of EV are evolving rapidly, there needs to be improved understanding of EV biogenesis and characteristics, the sorting of EV content and EV release as well as knowledge in the cellular uptake and transcriptional events in recipient cells. Through better understanding of mechanisms regulating EV release and cellular uptake, one may be able to manipulate the processes to a patient's advantage in the future. To address the gaps in current EV knowledge it is of critical importance to improve and standardize EV isolation and analysis techniques. A better methodology would also allow improved reproducibility of EV based results.

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References

1. WHO. *Global status report on noncommunicable diseases*. 2014.
2. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med*. 2006;3(11):e442.
3. Mackman N. Triggers, targets and treatments for thrombosis. *Nature*. 2008;451(7181):914–918.
4. Thygesen K, Alpert JS, Jaffe AS, et al. Third universal definition of myocardial infarction. *J Am Coll Cardiol*. 2012;60(16):1581–1598.
5. Carden DL, Granger DN. Pathophysiology of ischaemia-reperfusion injury. *J Pathol*. 2000;190(3):255–266.
6. Whayne TF. Epigenetics in the development, modification, and prevention of cardiovascular disease. *Mol Biol Rep*. 2014.
7. Felsenfeld G. A brief history of epigenetics. *Cold Spring Harb Perspect Biol*. 2014;6(1).
8. Webster AL, Yan MS, Marsden PA. Epigenetics and cardiovascular disease. *Can J Cardiol*. 2013;29(1):46–57.
9. Bird A. Perceptions of epigenetics. *Nature*. 2007;447(7143):396–398.
10. Abi Khalil C. The emerging role of epigenetics in cardiovascular disease. *Ther Adv Chronic Dis*. 2014;5(4):178–187.
11. Ordovás JM, Smith CE. Epigenetics and cardiovascular disease. *Nat Rev Cardiol*. 2010;7(9):510–519.
12. Shahbazian MD, Grunstein M. Functions of site-specific histone acetylation and deacetylation. *Annu Rev Biochem*. 2007;76:75–100.
13. Subramanian S, Bates S, Wright J, Espinoza-Delgado I, Piekarz R. Clinical Toxicities of Histone Deacetylase Inhibitors. *Pharmaceuticals*. 2010;3:2751–2767.
14. Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, Klein PS. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J Biol Chem*. 2001;276(39):36734–36741.
15. van der Pol E, Böing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol Rev*. 2012;64(3):676–705.
16. Yellon DM, Davidson SM. Exosomes: nanoparticles involved in cardioprotection? *Circ Res*. 2014;114(2):325–332.
17. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*. 2007;9(6):654–659.
18. Waldenström A, Gennebäck N, Hellman U, Ronquist G. Cardiomyocyte microvesicles contain DNA/RNA and convey biological messages to target cells. *PLoS One*. 2012;7(4):e34653.
19. Cai J, Wu G, Tan X, et al. Transferred BCR/ABL DNA from K562 extracellular vesicles causes chronic myeloid leukemia in immunodeficient mice. *PLoS One*. 2014;9(8):e105200.
20. Camussi G, Deregibus MC, Bruno S, Grange C, Fonsato V, Tetta C. Exosome/microvesicle-mediated epigenetic reprogramming of cells. *Am J Cancer Res*. 2011;1(1):98–110.
21. Lee Y, El Andaloussi S, Wood MJ. Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. *Hum Mol Genet*. 2012;21(R1):R125–134.
22. Oliver JJ, Webb DJ, Newby DE. Stimulated tissue plasminogen activator release as a marker of endothelial function in humans. *Arterioscler Thromb Vasc Biol*. 2005;25(12):2470–2479.
23. Kruithof EK, Dunoyer-Geindre S. Human tissue-type plasminogen activator. *Thromb Haemost*. 2014;112(2).
24. Fox KA, Robison AK, Knabb RM, Rosamond TL, Sobel BE, Bergmann SR. Prevention of coronary thrombosis with subthrombolytic doses of tissue-type plasminogen activator. *Circulation*. 1985;72(6):1346–1354.
25. Brommer EJ. The level of extrinsic plasminogen activator (t-PA) during clotting as a determinant of the rate of fibrinolysis; inefficiency of activators added afterwards. *Thromb Res*. 1984;34(2):109–115.
26. Hoylaerts M, Rijken DC, Lijnen HR, Collen D. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. *J Biol Chem*. 1982;257(6):2912–2919.
27. Devries S, Fox K, Robison A, Rodriguez R, Sobel B. Determinants of clearance of tissue-type plasminogen activator (t-PA) from the circulation. *Fibrinolysis*. 1987;17–21.
28. Hrafnkelsdottir T, Gudnason T, Wall U, Jern C, Jern S. Regulation of local availability of active tissue-type plasminogen activator in vivo in man. *J Thromb Haemost*. 2004;2(11):1960–1968.
29. Dellas C, Loskutoff DJ. Historical analysis of PAI-1 from its discovery to its potential role in cell motility and disease. *Thromb Haemost*. 2005;93(4):631–640.
30. Chandler WL, Alessi MC, Aillaud MF, Henderson P, Vague P, Juhan-Vague I. Clearance of tissue plasminogen activator (TPA) and TPA/plasminogen activator inhibitor type 1 (PAI-1) complex: relationship to elevated TPA antigen in patients with high PAI-1 activity levels. *Circulation*. 1997;96(3):761–768.

31. Jern C, Seeman-Lodding H, Biber B, Winsö O, Jern S. An experimental multiple-organ model for the study of regional net release/uptake rates of tissue-type plasminogen activator in the intact pig. *Thromb Haemost*. 1997;78(3):1150–1156.
32. Aspelin T, Eriksen M, Lindgaard AK, Lyberg T, Ilebekk A. Cardiac fibrinolytic capacity is markedly increased after brief periods of local myocardial ischemia, but declines following successive periods in anesthetized pigs. *J Thromb Haemost*. 2005;3(9):1947–1954.
33. Aspelin T, Eriksen M, Ilebekk A, Björkman JA, Lyberg T. Different cardiac tissue plasminogen activator release patterns by local stimulation of the endothelium and sympathetic nerves in pigs. *Blood Coagul Fibrinolysis*. 2012.
34. Wall U, Jern C, Jern S. High capacity for tissue-type plasminogen activator release from vascular endothelium in vivo. *J Hypertens*. 1997;15(12 Pt 2):1641–1647.
35. Osterlund B, Jern C, Seeman-Lodding H, et al. Intracoronary beta2 receptor activation induces dynamic local t-PA release in the pig. *Thromb Haemost*. 2003;90(5):796–802.
36. Osterlund B, Andersson B, Häggmark S, et al. Myocardial ischemia induces coronary t-PA release in the pig. *Acta Anaesthesiol Scand*. 2002;46(3):271–278.
37. Björkman JA, Jern S, Jern C. Cardiac sympathetic nerve stimulation triggers coronary t-PA release. *Arterioscler Thromb Vasc Biol*. 2003;23(6):1091–1097.
38. Stein CM, Brown N, Vaughan DE, Lang CC, Wood AJ. Regulation of local tissue-type plasminogen activator release by endothelium-dependent and endothelium-independent agonists in human vasculature. *J Am Coll Cardiol*. 1998;32(1):117–122.
39. Schneiderman J, Adar R, Savion N. Changes in plasmatic tissue-type plasminogen activator and plasminogen activator inhibitor activity during acute arterial occlusion associated with severe ischemia. *Thromb Res*. 1991;62(5):401–408.
40. Winnerkvist A, Wiman B, Valen G, Vaage J. Release of tissue plasminogen activator during reperfusion after different times of ischaemia in isolated, perfused rat hearts. *Thromb Res*. 1996;82(6):533–542.
41. Emeis JJ. Regulation of the acute release of tissue-type plasminogen activator from the endothelium by coagulation activation products. *Ann N Y Acad Sci*. 1992;667:249–258.
42. Giles AR, Nesheim ME, Herring SW, Hoogendoorn H, Stump DC, Heldebrant CM. The fibrinolytic potential of the normal primate following the generation of thrombin in vivo. *Thromb Haemost*. 1990;63(3):476–481.
43. Brown NJ, Gainer JV, Stein CM, Vaughan DE. Bradykinin stimulates tissue plasminogen activator release in human vasculature. *Hypertension*. 1999;33(6):1431–1435.
44. Jern C, Ladenvall P, Wall U, Jern S. Gene polymorphism of t-PA is associated with forearm vascular release rate of t-PA. *Arterioscler Thromb Vasc Biol*. 1999;19(2):454–459.

45. Ladenvall P, Johansson L, Jansson JH, et al. Tissue-type plasminogen activator -7,351C/T enhancer polymorphism is associated with a first myocardial infarction. *Thromb Haemost*. 2002;87(1):105–109.
46. Kathiresan S, Yang Q, Larson MG, et al. Common genetic variation in five thrombosis genes and relations to plasma hemostatic protein level and cardiovascular disease risk. *Arterioscler Thromb Vasc Biol*. 2006;26(6):1405–1412.
47. Hrafnkelsdóttir T, Wall U, Jern C, Jern S. Impaired capacity for endogenous fibrinolysis in essential hypertension. *Lancet*. 1998;352(9140):1597–1598.
48. Osterlund B, Jern S, Jern C, et al. Impaired myocardial t-PA release in patients with coronary artery disease. *Acta Anaesthesiol Scand*. 2008;52(10):1375–1384.
49. Van Guilder GP, Hoetzer GL, Smith DT, et al. Endothelial t-PA release is impaired in overweight and obese adults but can be improved with regular aerobic exercise. *Am J Physiol Endocrinol Metab*. 2005;289(5):E807–813.
50. Hrafnkelsdóttir T, Ottosson P, Gudnason T, Samuelsson O, Jern S. Impaired endothelial release of tissue-type plasminogen activator in patients with chronic kidney disease and hypertension. *Hypertension*. 2004;44(3):300–304.
51. Newby DE, McLeod AL, Uren NG, et al. Impaired coronary tissue plasminogen activator release is associated with coronary atherosclerosis and cigarette smoking: direct link between endothelial dysfunction and atherothrombosis. *Circulation*. 2001;103(15):1936–1941.
52. Robinson SD, Ludlam CA, Boon NA, Newby DE. Endothelial fibrinolytic capacity predicts future adverse cardiovascular events in patients with coronary heart disease. *Arterioscler Thromb Vasc Biol*. 2007;27(7):1651–1656.
53. Ulfhammer E, Larsson P, Karlsson L, et al. TNF-alpha mediated suppression of tissue type plasminogen activator expression in vascular endothelial cells is NF-kappaB- and p38 MAPK-dependent. *J Thromb Haemost*. 2006;4(8):1781–1789.
54. Larsson P, Ulfhammer E, Karlsson L, Bokarewa M, Wåhlander K, Jern S. Effects of IL-1beta and IL-6 on tissue-type plasminogen activator expression in vascular endothelial cells. *Thromb Res*. 2008;123(2):342–351.
55. Weil BR, Greiner JJ, DeSouza CA, Stauffer BL. Relation of C-reactive protein to endothelial fibrinolytic function in healthy adults. *Am J Cardiol*. 2011;108(11):1675–1679.
56. Speidl WS, Zeiner A, Nikfardjam M, et al. An increase of C-reactive protein is associated with enhanced activation of endogenous fibrinolysis at baseline but an impaired endothelial fibrinolytic response after venous occlusion. *J Am Coll Cardiol*. 2005;45(1):30–34.
57. Collen D, Lijnen HR. The tissue-type plasminogen activator story. *Arterioscler Thromb Vasc Biol*. 2009;29(8):1151–1155.
58. Zamarron C, Lijnen HR, Collen D. Influence of exogenous and endogenous tissue-type plasminogen activator on the lysability of clots in a plasma milieu in vitro. *Thromb Res*. 1984;35(3):335–345.

59. Arts J, Lansink M, Grimbergen J, Toet KH, Kooistra T. Stimulation of tissue-type plasminogen activator gene expression by sodium butyrate and trichostatin A in human endothelial cells involves histone acetylation. *Biochem J*. 1995;310 (Pt 1):171–176.
60. Dunoyer-Geindre S, Kruithof EK. Epigenetic control of tissue-type plasminogen activator synthesis in human endothelial cells. *Cardiovasc Res*. 2011;90(3):457–463.
61. Larsson P, Bergh N, Lu E, et al. Histone deacetylase inhibitors stimulate tissue-type plasminogen activator production in vascular endothelial cells. *J Thromb Thrombolysis*. 2013;35(2):185–192.
62. Göttlicher M, Minucci S, Zhu P, et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J*. 2001;20(24):6969–6978.
63. Karén J, Rodriguez A, Friman T, Dencker L, Sundberg C, Scholz B. Effects of the histone deacetylase inhibitor valproic acid on human pericytes in vitro. *PLoS One*. 2011;6(9):e24954.
64. Wheler JJ, Janku F, Falchook GS, et al. Phase I study of anti-VEGF monoclonal antibody bevacizumab and histone deacetylase inhibitor valproic acid in patients with advanced cancers. *Cancer Chemother Pharmacol*. 2014;73(3):495–501.
65. Causey MW, Miller S, Hoffer Z, et al. Beneficial effects of histone deacetylase inhibition with severe hemorrhage and ischemia-reperfusion injury. *J Surg Res*. 2013;184(1):533–540.
66. Munster P, Marchion D, Bicaku E, et al. Clinical and biological effects of valproic acid as a histone deacetylase inhibitor on tumor and surrogate tissues: phase I/II trial of valproic acid and epirubicin/FEC. *Clin Cancer Res*. 2009;15(7):2488–2496.
67. Larsson P, Ulfhammer E, Magnusson M, et al. Role of histone acetylation in the stimulatory effect of valproic Acid on vascular endothelial tissue-type plasminogen activator expression. *PLoS One*. 2012;7(2):e31573.
68. Gupta S, Knowlton AA. HSP60 trafficking in adult cardiac myocytes: role of the exosomal pathway. *Am J Physiol Heart Circ Physiol*. 2007;292(6):H3052–3056.
69. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol*. 2013;200(4):373–383.
70. Lötvall J, Hill AF, Hochberg F, et al. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. *J Extracell Vesicles*. 2014;3:26913.
71. Escola JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O, Geuze HJ. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem*. 1998;273(32):20121–20127.

72. Lai RC, Chen TS, Lim SK. Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. *Regen Med*. 2011;6(4):481–492.
73. Kahlert C, Melo SA, Protopopov A, et al. Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. *J Biol Chem*. 2014;289(7):3869–3875.
74. Raiborg C, Rusten TE, Stenmark H. Protein sorting into multivesicular endosomes. *Curr Opin Cell Biol*. 2003;15(4):446–455.
75. Eldh M, Ekström K, Valadi H, et al. Exosomes communicate protective messages during oxidative stress; possible role of exosomal shuttle RNA. *PLoS One*. 2010;5(12):e15353.
76. de Jong OG, Verhaar MC, Chen Y, et al. Cellular stress conditions are reflected in the protein and RNA content of endothelial cell-derived exosomes. *J Extracell Vesicles*. 2012;1.
77. Waldenström A, Ronquist G. Role of exosomes in myocardial remodeling. *Circ Res*. 2014;114(2):315–324.
78. Guescini M, Genedani S, Stocchi V, Agnati LF. Astrocytes and Glioblastoma cells release exosomes carrying mtDNA. *J Neural Transm*. 2010;117(1):1–4.
79. Ronquist KG, Ronquist G, Carlsson L, Larsson A. Human prostasomes contain chromosomal DNA. *Prostate*. 2009;69(7):737–743.
80. Ronquist GK, Larsson A, Stavreus-Evers A, Ronquist G. Prostasomes are heterogeneous regarding size and appearance but affiliated to one DNA-containing exosome family. *Prostate*. 2012;72(16):1736–1745.
81. Olsson I, Ronquist G. Nucleic acid association to human prostasomes. *Arch Androl*. 1990;24(1):1–10.
82. Balaj L, Lessard R, Dai L, et al. Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. *Nat Commun*. 2011;2:180.
83. Thakur BK, Zhang H, Becker A, et al. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. *Cell Res*. 2014;24(6):766–769.
84. Lázaro-Ibáñez E, Sanz-Garcia A, Visakorpi T, et al. Different gDNA content in the subpopulations of prostate cancer extracellular vesicles: apoptotic bodies, microvesicles, and exosomes. *Prostate*. 2014;74(14):1379–1390.
85. Cai J, Han Y, Ren H, Chen C, He D, Zhou L, et al. Extracellular vesicle-mediated transfer of donor genomic DNA to recipient cells is a novel mechanism for genetic influence between cells. *J Mol Cell Biol*. 2013;5(4):227–38.
86. Lee TH, Chennakrishnaiah S, Audemard E, Montermini L, Meehan B, Rak J. Oncogenic ras-driven cancer cell vesiculation leads to emission of double-stranded DNA capable of interacting with target cells. *Biochem Biophys Res Commun*. 2014;451(2):295–301.
87. Gennebäck N, Hellman U, Malm L, et al. Growth factor stimulation of cardiomyocytes induces changes in the transcriptional contents of secreted exosomes. *J Extracell Vesicles*. 2013;2.

88. Malik ZA, Kott KS, Poe AJ, et al. Cardiac myocyte exosomes: stability, HSP60, and proteomics. *Am J Physiol Heart Circ Physiol*. 2013;304(7):H954–965.
89. Lai RC, Arslan F, Lee MM, et al. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res*. 2010;4(3):214–222.
90. Chen L, Wang Y, Pan Y, et al. Cardiac progenitor-derived exosomes protect ischemic myocardium from acute ischemia/reperfusion injury. *Biochem Biophys Res Commun*. 2013;431(3):566–571.
91. Arslan F, Lai RC, Smeets MB, et al. Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. *Stem Cell Res*. 2013;10(3):301–312.
92. Ibrahim AG, Cheng K, Marbán E. Exosomes as critical agents of cardiac regeneration triggered by cell therapy. *Stem Cell Reports*. 2014;2(5):606–619.
93. Barile L, Lionetti V, Cervio E, et al. Extracellular vesicles from human cardiac progenitor cells inhibit cardiomyocyte apoptosis and improve cardiac function after myocardial infarction. *Cardiovasc Res*. 2014;103(4):530–541.
94. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation*. 1986;74(5):1124–1136.
95. Bolli R. The late phase of preconditioning. *Circ Res*. 2000;87(11):972–983.
96. Yellon DM, Downey JM. Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol Rev*. 2003;83(4):1113–1151.
97. Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. *Circulation*. 1993;87(3):893–899.
98. Hausenloy DJ, Yellon DM. Remote ischaemic preconditioning: underlying mechanisms and clinical application. *Cardiovasc Res*. 2008;79(3):377–386.
99. Shi W, Vinten-Johansen J. Endogenous cardioprotection by ischaemic postconditioning and remote conditioning. *Cardiovasc Res*. 2012;94(2):206–216.
100. Giricz Z, Varga ZV, Baranyai T, et al. Cardioprotection by remote ischemic preconditioning of the rat heart is mediated by extracellular vesicles. *J Mol Cell Cardiol*. 2014;68:75–78.
101. Feng Y, Huang W, Wani M, Yu X, Ashraf M. Ischemic preconditioning potentiates the protective effect of stem cells through secretion of exosomes by targeting Mecp2 via miR-22. *PLoS One*. 2014;9(2):e88685.
102. Yamaguchi T, Izumi Y, Nakamura Y, et al. Repeated remote ischemic conditioning attenuates left ventricular remodeling via exosome-mediated intercellular communication on chronic heart failure after myocardial infarction. *Int J Cardiol*. 2015;178:239–246.
103. Vicencio M, Zheng Y, Das D, et al. *Plasma exosomes from rats and humans protect the myocardium from ischemia-reperfusion injury*. Cardiovascular Research 2014.

104. Liu J, Yellon D, Davidson S. Evaluating early and delayed cardioprotection by plasma exosomes in simulated ischaemia–reperfusion injury. *Bioscience-Horizons*. 2015;8:1–11.
105. Swindle MM, Smith AC, Hepburn BJ. Swine as models in experimental surgery. *J Invest Surg*. 1988;1(1):65–79.
106. Häggmark S, Biber B, Sjödin JG, Winsö O, Gustavsson B, Reiz S. The continuous thermodilution method for measuring high blood flows. *Scand J Clin Lab Invest*. 1982;42(4):315–321.
107. Kavianipour M, Ronquist G, Wikström G, Waldenström A. Ischaemic preconditioning alters the energy metabolism and protects the ischaemic myocardium in a stepwise fashion. *Acta Physiol Scand*. 2003;178(2):129–137.
108. Klöcking HP, Hoffmann A. Inhibition of thrombin-induced tissue-type plasminogen activator release by agents which increase intracellular cyclic AMP. *Thromb Res*. 1990;59(4):789–792.
109. Veenstra J, Kluft C, Ockhuizen TH, vd Pol H, Wedel M, Schaafsma G. Effects of moderate alcohol consumption on platelet function, tissue-type plasminogen activator and plasminogen activator inhibitor. *Thromb Haemost*. 1990;63(3):345–348.
110. Eliasson M, Asplund K, Evrin PE. Regular leisure time physical activity predicts high activity of tissue plasminogen activator: The Northern Sweden MONICA Study. *Int J Epidemiol*. 1996;25(6):1182–1188.
111. Van Guilder GP, Hoetzer GL, Greiner JJ, Stauffer BL, DeSouza CA. Acute and chronic effects of vitamin C on endothelial fibrinolytic function in overweight and obese adult humans. *J Physiol*. 2008;586(14):3525–3535.
112. Kluft C, Kooistra T, Veenstra J, Schaafsma G. Effects of coffee and caffeine on tissue-type plasminogen activator and its fast-acting inhibitor, PAI-1, in vivo and in vitro. *Fibrinolysis*. 1990;4:74–75.
113. Jern C, Selin L, Jern S. Application of the perfused-forearm model to study release mechanisms of tissue-type plasminogen activator in man. *Fibrinolysis*. 1994;8:13–15.
114. Wilkinson I, Webb D. Venous occlusion plethysmography in cardiovascular research: methodology and clinical applications. *J Clin Pharmacol*. 2001;52:631–646.
115. Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterzation of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol*. 2006;Chapter 3:Unit 3.22.
116. Van Deun J, Mestdagh P, Sormunen R, et al. The impact of disparate islation methods for extracellular vesicles on downstream RNA profiling. *J Extracell Vesicles*. 2014;3.
117. Witwer KW, Buzás EI, Bemis LT, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles*. 2013;2.

118. Ness SA. Basic microarray analysis: strategies for successful experiments. *Methods Mol Biol*. 2006;316:13 – 33.
119. Huang dW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4(1):44 – 57.
120. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol*. 2004;3:Article3.
121. Smyth G. Limma: linear models for microarray data. In: R. Gentleman VC, S.Dudoit, R. Irizarry, W. Huber, ed. *Bioinformatics and Computational Biology Solutions using R and Bioconductor:* Springer, New York; 2005:397 – 420.
122. Benjamini YH. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B*. 1995:289 – 300.
123. Heusch G, Musiolik J, Gedik N, Skyschally A. Mitochondrial STAT3 activation and cardioprotection by ischemic postconditioning in pigs with regional myocardial ischemia/reperfusion. *Circ Res*. 2011;109(11):1302 – 1308.
124. Kaga S, Zhan L, Altaf E, Maulik N. Glycogen synthase kinase-3beta/beta-catenin promotes angiogenic and anti-apoptotic signaling through the induction of VEGF, Bcl-2 and survivin expression in rat ischemic preconditioned myocardium. *J Mol Cell Cardiol*. 2006;40(1):138 – 147.
125. Bolli R, Shinmura K, Tang XL, Kodani E, Xuan YT, Guo Y, et al. Discovery of a new function of cyclooxygenase (COX)-2: COX-2 is a cardioprotective protein that alleviates ischemia/reperfusion injury and mediates the late phase of preconditioning. *Cardiovasc Res*. 2002;55(3):506 – 19.
126. Miura T, Tanno M. Mitochondria and GSK-3beta in cardioprotection against ischemia/reperfusion injury. *Cardiovasc Drugs Ther*. 2010;24(3):255 – 263.
127. Fauconnier J, Roberge S, Saint N, Lacampagne A. Type 2 ryanodine receptor: a novel therapeutic target in myocardial ischemia/reperfusion. *Pharmacol Ther*. 2013;138(3):323 – 332.
128. Bolli R, Stein AB, Guo Y, et al. A murine model of inducible, cardiac-specific deletion of STAT3: its use to determine the role of STAT3 in the upregulation of cardioprotective proteins by ischemic preconditioning. *J Mol Cell Cardiol*. 2011;50(4):589 – 597.
129. Löscher W. Valproate: a reappraisal of its pharmacodynamic properties and mechanisms of action. *Prog Neurobiol*. 1999;58(1):31 – 59.
130. Cho KS, Kwon KJ, Choi CS, et al. Valproic acid induces astrocyte-dependent neurite outgrowth from cultured rat primary cortical neuron via modulation of tPA/PAI-1 activity. *Glia*. 2013;61(5):694 – 709.

131. Saluveer O, Larsson P, Ridderstråle W, Hrafnkelsdóttir TJ, Jern S, Bergh N. Profibrinolytic effect of the epigenetic modifier valproic Acid in man. *PLoS One*. 2014;9(10):e107582.
132. Brogren H, Sihlbom C, Wallmark K, et al. Heterogeneous glycosylation patterns of human PAI-1 may reveal its cellular origin. *Thromb Res*. 2008;122(2):271 – 281.
133. Booth NA, Simpson AJ, Croll A, Bennett B, MacGregor IR. Plasminogen activator inhibitor (PAI-1) in plasma and platelets. *Br J Haematol*. 1988;70(3):327 – 333.
134. Olesen JB, Abildstrøm SZ, Erdal J, et al. Effects of epilepsy and selected antiepileptic drugs on risk of myocardial infarction, stroke, and death in patients with or without previous stroke: a nationwide cohort study. *Pharmacoepidemiol Drug Saf*. 2011;20(9):964 – 971.
135. Olesen JB, Hansen PR, Abildstrøm SZ, et al. Valproate attenuates the risk of myocardial infarction in patients with epilepsy: a nationwide cohort study. *Pharmacoepidemiol Drug Saf*. 2011;20(2):146 – 153.
136. Dregan A, Charlton J, Wolfe CD, Gulliford MC, Markus HS. Is sodium valproate, an HDAC inhibitor, associated with reduced risk of stroke and myocardial infarction? A nested case-control study. *Pharmacoepidemiol Drug Saf*. 2014;23(7):759 – 767.
137. Van Lint C, Emiliani S, Verdin E. The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. *Gene Expr*. 1996;5(4 – 5):245 – 253.
138. Xu WS, Parmigiani RB, Marks PA. Histone deacetylase inhibitors: molecular mechanisms of action. *Oncogene*. 2007;26(37):5541 – 5552.
139. Swindle MM, Makin A, Herron AJ, Clubb FJ, Frazier KS. Swine as models in biomedical research and toxicology testing. *Vet Pathol*. 2012;49(2):344 – 356.
140. Osterlund B, Holmgren A, Häggmark S, et al. Surgical stress induces acute coronary release of tissue-type plasminogen activator in the pig. *Acta Anaesthesiol Scand*. 2000;44(10):1226 – 1231.
141. Hoetzer GL, Stauffer BL, Irmiger HM, Ng M, Smith DT, DeSouza CA. Acute and chronic effects of oestrogen on endothelial tissue-type plasminogen activator release in postmenopausal women. *J Physiol*. 2003;551(Pt 2):721 – 728.
142. O'Gara PT, Kushner FG, Ascheim DD, et al. 2013 ACCF/AHA guideline for the management of ST-elevation myocardial infarction: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *Circulation*. 2013;127(4):e362 – 425.
143. Jern C, Selin L, Jern S. In vivo release of tissue-type plasminogen activator across the human forearm during mental stress. *Thromb Haemost*. 1994;72(2):285 – 291.
144. Newby DE, Wright RA, Ludlam CA, Fox KA, Boon NA, Webb DJ. An in vivo model for the assessment of acute fibrinolytic capacity of the endothelium. *Thromb Haemost*. 1997;78(4):1242 – 1248.

145. Katan-Khaykovich Y, Struhl K. Dynamics of global histone acetylation and deacetylation in vivo: rapid restoration of normal histone acetylation status upon removal of activators and repressors. *Genes Dev*. 2002;16(6):743–752.
146. Clayton AL, Hazzalin CA, Mahadevan LC. Enhanced histone acetylation and transcription: a dynamic perspective. *Mol Cell*. 2006;23(3):289–296.
147. Björkman JA, 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. *Thromb Res*. 2005;116(6):519–524.
148. Lenfors S, Gustafsson D. New model for in vivo studies of pharmacological interventions with endogenous fibrinolysis: effects of thrombin inhibitors. *Semin Thromb Hemost*. 1996;22(4):335–342.
149. Bolli R, Dawn B, Xuan YT. Role of the JAK-STAT pathway in protection against myocardial ischemia/reperfusion injury. *Trends Cardiovasc Med*. 2003;13(2):72–79.
150. Rodriguez DA, Tapia JC, Fernandez JG, et al. Caveolin-1-mediated suppression of cyclooxygenase-2 via a beta-catenin-Tcf/Lef-dependent transcriptional mechanism reduced prostaglandin E2 production and survivin expression. *Mol Biol Cell*. 2009;20(8):2297–2310.
151. Guo Y, Tukaye DN, Wu WJ, et al. The COX-2/PGI2 receptor axis plays an obligatory role in mediating the cardioprotection conferred by the late phase of ischemic preconditioning. *PLoS One*. 2012;7(7):e41178.
152. Shinmura K, Tang XL, Wang Y, et al. Cyclooxygenase-2 mediates the cardioprotective effects of the late phase of ischemic preconditioning in conscious rabbits. *Proc Natl Acad Sci U S A*. 2000;97(18):10197–10202.
153. Loor G, Schumacker PT. Role of hypoxia-inducible factor in cell survival during myocardial ischemia-reperfusion. *Cell Death Differ*. 2008;15(4):686–690.
154. Murphy E, Steenbergen C. Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiol Rev*. 2008;88(2):581–609.
155. Fauconnier J, Meli AC, Thireau J, et al. Ryanodine receptor leak mediated by caspase-8 activation leads to left ventricular injury after myocardial ischemia-reperfusion. *Proc Natl Acad Sci U S A*. 2011;108(32):13258–13263.
156. Waldenström A, Ronquist G, Aberg AM, et al. Ischaemic preconditioning reduces myocardial calcium overload in coronary-occluded pig hearts shown by continuous in vivo assessment using microdialysis. *Clin Physiol Funct Imaging*. 2012;32(2):133–138.
157. Zucchi R, Ronca F, Ronca-Testoni S. Modulation of sarcoplasmic reticulum function: a new strategy in cardioprotection? *Pharmacol Ther*. 2001;89(1):47–65.
158. Jeppesen DK, Hvam ML, Primdahl-Bengtson B, et al. Comparative analysis of discrete exosome fractions obtained by differential centrifugation. *J Extracell Vesicles*. 2014;3:25011.

Appendix I–IV