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INSIGHT ON THYMOSIN BETA 4 IN ANIMAL MODELS OF MYOCARDIAL INJURY

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ABSTRACT

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The use of small and large animal models is vital in the clinical translation of experimental data in cardiovascular research. Thymosin beta 4 is a peptide which has shown good cardioprotective potential in several *in vitro* and animal experiments. The peptide increases cell survival, angiogenesis and progenitor cell migration while it reduces inflammation and cell death. In small animal models for myocardial infarction it limits infarct size and improves cardiac function. It has also shown promising effect in pig models for myocardial injury. In this thesis, we aimed to determine the effect of the peptide in mouse models for myocardial infarction and anthracycline-induced cardiomyopathy and in a pig model for global ischemia-reperfusion injury. We focused on improvement in cardiac functional outcome by performing echocardiography, magnetic resonance and positron emission tomography imaging. The underlying mechanism were investigated by measuring cell death, inflammation and gene-expression analysis. After myocardial infarction thymosin beta 4 treatment may have reduced infarct expansion and left ventricle remodeling with improvement in left ventricle function. Treatment was associated with upregulation of chitinase 3-like-1, a novel protein involved in fibrosis and inflammation. We also observed increased activity of the cardioprotective enzyme CD73. Anthracycline caused high mortality with limited change in cardiac contractility and was therefore not considered suitable for inducing experimental cardiac dysfunction. Thymosin beta 4 did not attenuate global myocardial ischemia-reperfusion injury nor did it influence myocardial cell death or inflammation. The cardioprotective effect seen in mice could not be demonstrated in pigs and further therapeutic evaluation is therefore needed.

Keywords: thymosin beta 4, cardioprotection, myocardial infarction, ischemia-reperfusion injury, anthracycline-induced cardiomyopathy, echocardiography, cardiac magnetic resonance imaging, inflammation, apoptosis, chitinase 3-like-1, CD73

SAMMANFATTNING

Christoffer Stark
THYMOSIN BETA 4 OCH FÖRSÖKSDJURSMODELLER FÖR HJÄRTMUSKELSKADA

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Små och stora försöksdjursmodeller är viktiga för att utveckla prekliniska resultat inom kardiovaskulär forskning. Thymosin beta 4 är en peptid som visat kardioprotektiva egenskaper i flera *in vitro* och försöksdjursexperiment. Peptiden förbättrar cellernas överlevnad efter olika typer av stress, hämmar inflammation och ökar utvecklandet av nya blodkärl. Efter behandling för hjärtinfarkt minskar peptiden i små försöksdjursmodeller infarktstorleken och leder till förbättrad hjärtfunktion. Den har också visat lovande resultat i grismodeller för hjärtmuskelskada. I den här avhandlingens delarbeten undersöktes peptidens effekter efter hjärtinfarkt och antracyklin-inducerad hjärtsvikt med hjälp av musmodeller och efter iskemi-reperfusionsskada på hjärtat i en grismodell. Vi utförde avbildningsundersökningar för att bestämma peptidens inverkan på hjärtats funktion efter hjärtskadorna och undersökte också mängden av hjärtcellsdöd, inflammation och förändringar i hjärtats genekspression. Thymosin beta 4 behandlingen förbättrade möjligtvis hjärtfunktionen och minskade infarktområdets utvidgning och hjärtats remodellering. Chitinase 3-like-1, ett protein som påverkar inflammation och produktionen av bindvävnad upreglerades efter behandlingen. Aktiviteten av det kardioprotektiva entsymet CD73 var också förhöjt. Dödligheten hos antracyklinbehandlade möss var hög och hjärtfunktionen var inte märkbart nedsatt. Därför ansågs behandlingen inte vara lämplig för att orsaka hjärtsvikt. Thymosin beta 4 hade ingen inverkan på hjärtat efter iskemi-reperfusionskada. Behandlingen minskade inte heller mängden av celldöd eller inflammation. Den kardioprotektiva effekten hos möss kunde därmed inte påvisas i grismodellen och ytterligare terapeutisk utvärdering av peptiden är nödvändig i framtiden.

Nyckelord: thymosin beta 4, kardioprotektion, hjärtinfarkt, iskemi-reperfusionsskada, antracyklin-inducerad hjärtsvikt, ultraljudskardiografi, magnetresonanstomografi, inflammation, apoptos, chitinase 3-like-1, CD 73

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ABBREVIATIONS

ADP	adenosine diphosphate
ADPases	ADP hydrolyzing enzymes
AK	adenylate kinase
Akt	protein kinase B
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATPases	ATP hydrolyzing enzymes
Bcl-2	B-cell lymphoma 2
CABG	coronary artery bypass grafting
CAD	coronary artery disease
CBF	coronary blood flow
ccdc80	coiled-coil domain containing 80
CD209f	cluster of differentiation 209f
CD68	cluster of differentiation 68
CD73	5'-endonucleotidase
cGMP	cyclic guanosine monophosphate
Ch3l1	chitinase 3-like-1
CLP	chitinase-like protein
CMR	cardiac magnetic resonance imaging
CPB	cardiopulmonary bypass
cTnI/TnT	cardiac troponin I/T
DAMPs	danger associated molecular patterns
DNA	deoxyribonucleic acid
EDV	end-diastolic volume
EF	ejection fraction
EIA	enzyme immunoassay
eNOS	endothelial nitric oxide synthase
Erk 1/2	extracellular signal related kinase 1/2
ESV	end-systolic volume
FAK	focal adhesion kinase
FS	fractional shortening
FVIIa	activated factor VII
FXa	activated factor X
FXIIIa	activated factor XIII
GpIIb/IIIa	glycoprotein IIb/IIIa
HF	heart failure
HGF	hepatocyte growth factor
IFN-γ	interferon gamma

IL-10	interleukin 10
IL-18	interleukin 18
IL-1α	interleukin- 1 alpha
IL-1β	interleukin 1 beta
IL-6	interleukin 6
I-RI	ischemia-reperfusion injury
Ki-67	cell proliferation marker
LAD	left anterior descending coronary artery
LCX	left circumflex coronary artery
L-NNA	NG-nitro-L-arginine
LV	left ventricle
LVIDs/d	left ventricle internal diameter in systole/diastole
MBF	myocardial blood flow
Mek 1/2	mitogen activated protein kinase 1/2
MI	myocardial infarction
mRNA	messenger ribonucleic acid
NDPK	nucleoside diphosphate kinase
NF-κB	nuclear factor kappa B
NO	nitric oxide
PAR2	peroxisome activated receptor 2
PBS	phosphate buffered saline
PET	positron emission tomography
PI3K	phosphoinositol 3-kinase
PIP complex	PINCH-ILK-alpha parvin complex
PLD	pegylated liposomal doxorubicin
RAA	renin-angiotensin-aldosterone
RCA	right coronary artery
RISK	reperfusion injury salvage kinase
RNA	ribonucleic acid
ROS	reactive oxygen species
RV	right ventricle
SAFE	survival activating factor enhancement
SIRS	systemic inflammatory response syndrome
Stard10	start domain containing 10
TB4	thymosin beta 4
TF	tissue factor
TGF-β	transforming growth factor beta
Th2 cell	T-helper 2 lymphocyte

TNF-α	tumor necrosis factor alpha
tPA	tissue plasminogen activator
TUNEL	terminal uDTP nick-end labeling
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell
vWF	von Willebrand factor

LIST OF ORIGINAL PUBLICATIONS

I. Stark C, Helenius M, Taimen P, Kentala R, Saraste A, Alastalo T-P, Savunen T, Koskenvuo J. Thymosin beta 4 treatment improves left ventricular function after myocardial infarction and is related to up-regulation of chitinase-3-like-1 in mice. Translational medicine communications. 2016;1:8.

II. Stark C, Taimen P, Savunen T, Koskenvuo J. Cardiac function, fibrosis and survival in a mouse model for pegylated liposomal doxorubicin-induced cardiomyopathy and thymosin beta 4 treatment. Manuscript.

III. Stark C, Tarkia M, Kentala R, Malmberg M, Vähäsilta T, Savo M, Hynninen V-V, Helenius M, Ruohonen S, Jalkanen J, Taimen P, Alastalo T-P, Saraste A, Knuuti J, Savunen T, Koskenvuo J. Systemic dosing of thymosin beta 4 before and after ischemia does not attenuate global myocardial ischemia-reperfusion injury in pigs. Frontiers in pharmacology. 2016;7:115.

1 INTRODUCTION

The prevalence of stable coronary artery disease (CAD) is reported to be 4-7 % in younger populations and 10-14 % in ages over 65. The phenotype and disease presentation is often different between younger and older patients due to more common microvessel involvement in younger age groups. Chest pain or angina is present in 1-4 % of patients within the same population. The annual mortality rate with stable CAD is 1.2 – 2.4 % and in patients with several comorbidities up to 3.8 % [Montalescot et al 2013]. Patients with acute myocardial infarction (MI) usually present with specific electrocardiographic changes categorizing them into infarct groups with non-ST-elevation or ST-elevation. ST-elevation is related to transmural injury of the myocardium. The short-term survival in NSTEMI patients is better but this difference is equalized after 1-2 years due to different patient characteristics at onset of MI [Roffi et al 2016].

Heart failure (HF) develops due to structural or functional cardiac abnormalities and is often secondary to ischemia or MI. Anti-cancer drug cardiotoxicity is another important cause of HF. The prevalence of HF in adult populations in developed countries is 1-2 %. In age groups over 70 years the prevalence exceeds 10 %. Mortality in HF is high. Stable patients have a 1-year mortality risk of 7 % while the risk for hospitalized patients is 17 % [Ponikowski et al 2016, Zamorano et al 2017].

In cardiac surgery, the use of cardiopulmonary bypass (CPB) and aortic cross-clamping causes cardiac damage through ischemia-reperfusion injury (I-RI). Reperfusion injury is also seen after revascularization procedures after MI. The precise impact of I-RI on patient outcome is difficult to determine due to many confounding factors but it is considered to increase patient mortality and morbidity [Hausenloy et al 2016].

All the above mentioned clinical scenarios are possible to reproduce in experimental settings which is useful in cardiovascular research and in the development of novel therapeutics. Standardizing experimental animal models and translating the outcome in these models into clinical practice is challenging. It is however necessary to determine the underlying biological mechanisms and efficacy of new treatments in order to proceed into clinical studies. An important step in this process is to reproduce experiments, performed in *in vitro* conditions also in small and large animal models. After ischemic insult to the heart the therapeutic approaches consist of antithrombotic therapy, lowering of myocardial oxygen consumption and early revascularization. There is no effective therapy replacing already lost or scarred heart tissue. Therefore, more effective cardioprotective therapies are necessary [Bell et al 2016].

Thymosin beta 4 (TB4) is a naturally occurring peptide expressed in most cell types which has been shown to offer cardioprotection after different types of cardiac insult in several experimental studies. The protective effects are related to increased cell survival, the formation of new blood vessels and activation of progenitor cells involved in myocardial healing after injury. A decrease in cell death and inflammation has also been observed with TB4 treatment [Goldstein et al 2012].

In the studies of this thesis we investigated the effects of TB4 in two different mouse models and in one pig model of myocardial injury. The primary objective was to demonstrate functional cardiac improvement after TB4 therapy by using different imaging modalities and to investigate its influence on cell death, inflammation and fibrosis. We also wanted to identify possible new therapeutic targets of the peptide.

2 REVIEW OF LITERATURE

2.1 Comparison of human, murine and porcine hearts

2.1.1 The human heart

The adult human heart weighs approximately 250-350 g and beats 60-100 times per minute. It is a hollow muscle pump that is responsible for the perfusion of peripheral tissues. The heart is composed of a left and a right side. The left ventricle (LV) pumps oxygenated blood returning via the pulmonary veins and left atrium to the rest of the body. The right ventricle (RV) receives deoxygenated blood via the venae cavae and the right atrium and pumps the blood through the pulmonary trunk and arteries to the lungs. The atria and ventricles are separated by a muscular septum. The direction of blood flow within the heart is controlled by four valve structures. The atria and ventricles are separated on the left side by the mitral valve and on the right side by the tricuspid valve. The LV and aorta are separated by the aortic valve and the RV and pulmonary trunk by the pulmonary valve [Zhong 2015].

The cellular composition of the heart consists of heart muscle cells (30 %), endothelial cells (45 %), fibroblasts (10 %), hematopoietic cells (5 %) and other smaller cell populations [Pinto et al 2016]. The heart muscle cells are interconnected by tight intercalated discs and form myofibrillar structures that compose the heart muscle itself. The inner surface of the heart muscle wall is lined with a thin endocardial cell layer. The outmost part of the muscular wall is the epicardium and between these structures lies the myocardium. The heart and proximal parts of the great vessels entering and leaving the heart are covered by a fibrous sac, a two-sided structure with visceral (epicardium) and parietal (pericardium) surfaces. The pericardial space that lies between these layers contains small amounts of pericardial fluid [Zhong 2015].

The myocardium receives its blood supply from the coronary arteries. The coronary arteries arise from the proximal aorta, distal to the aortic valve at the sinuses of Valsalva. Normally two main coronary arteries are present, the left and the right. The left main coronary artery divides into the left anterior descending (LAD) and left circumflex (LCX) arteries. The LAD runs distally along the interventricular groove and gives of branches to the anterolateral wall of the LV and to the apex. Its septal branches supply the anterior two thirds of the interventricular septum. After debranching from the left main coronary artery the LCX runs inferiorly to the left atrial appendage and supplies parts of the lateral and posterior parts of the LV and left atrium. The right coronary artery (RCA) runs proximally in the right atrioventricular groove and gives off blood supply to the right atrium, most parts

of the right ventricle and posterior interventricular septum. It also supplies the sinoatrial and atrioventricular nodes and small parts of the LV. The epicardial cardiac veins drain into the coronary sinus that runs on the posterior surface of the heart emptying into the right atrium through the coronary sinus. Some of the veins drain directly into the right side of the heart through the anterior cardiac veins or venae cordis minimae. The hearts electrical activity arises in the sinoatrial node situated at the junction between the right atrium and superior vena cava. After excitement of the atria, the electrical impulse continues through the atrioventricular node, located in the lower right atrial septum. The impulse then continues down the bundle of His and Purkinje fibers from the septum to excite both ventricles [Zhong 2015].

2.1.2 The mouse heart

The mouse heart weighs 100-200 mg and beats 500-800 times per minute. It is ellipsoidal in shape compared to the three-sided pyramid shape of human hearts. In contrast to humans, mice have both left- and right-sided superior (anterior) venae cavae. The pulmonary vein circulation is also different as the pulmonary veins drain into the left atrium through a single foramen in the dorsal wall of the left atrium via a pulmonary confluence. In mice the coronary arteries are mostly intramural and hardly visible. The myocardium has a well-organized capillary network. The LCX is small or rudimentary and most of the LV is supplied by the LAD. A large septal branch often arises from the proximal LAD. Distally it is divided into anterior and lateral segments. The RCA supplies mainly the right atrium and ventricle and its course is highly variable. Most of the venous return drains through the left cardiac vein into the groove between the left and right superior venae cavae. The electrical conduction of the mouse heart is similar to humans, mice however lack Purkinje fibers and the ventricles are probably excited by direct myocyte conduction [Gan et al 2004].

2.1.3 The porcine heart

The pig has a valentine shaped heart and it beats 91-167 times per minute. The heart weight to body weight ratio in commonly used 20-30 kg pigs is the same as in humans. Pigs are very excitable and their systemic and pulmonary blood pressures rise easily. The pig is also prone to arrhythmia. The difference in heart orientation inside the chest compared to humans is mainly related to stance. The heart is rotated counter-clockwise with the right atrium and ventricle facing cranially. The pig has only two pulmonary veins and the left azygos vein drains directly into the coronary sinus. Both ventricles are coarsely trabeculated with septomarginal trabeculae containing Purkinje fibers. These are found especially in the RV wall. The left to right ventricle wall thickness ratio is greater than in humans. The coronary circulation is strikingly similar to humans and pigs have very sparse formation

of native collateral circulation. The atrial myocardium of pigs contains numerous nerves and the function of the sinoatrial node and atrioventricular conduction system differs from that of humans. The pigs' conduction system is considered neuromyogenic compared to myogenic in humans. The similarities in anatomy, circulation and hemodynamics between pigs and humans are the reason why pigs are considered superior to other animal models in cardiovascular research [Lelovas et al 2014, Crick et al 1998].

2.2 Vasomotor control of coronary arteries

The coronary artery resistance vessels are divided according to their size into large arteries (diameter > 400 µm), small arteries (diameter 100-400 µm) and arterioles (diameter < 100 µm). The large arteries are conduit vessels responsible for only 5 % of the coronary vascular resistance. The small intramural arteries cause 40 % of the resistance and the arterioles and microvessels are thereby responsible for most of the resistance. The oxygen extraction in the myocardial vasculature is almost maximal (60-80 %) already in the resting state and therefore the increase in oxygen demand must be compensated for by an increase in coronary blood flow (CBF). During stress the myocardial blood flow can increase 5-fold compared to rest. CBF is relatively constant with different perfusion pressures when myocardial oxygen consumption is kept constant. This is accomplished by change in vasomotor tone of the resistance vessels and is referred to as autoregulation. The lower end of this regulatory area is reached with a perfusion pressure leading to maximal vasodilation of the microvessels. When the pressure falls below the autoregulatory threshold myocardial ischemia develops. During stress the lower end of this threshold is elevated due to increased myocardial oxygen consumption (due to increase in heart rate, afterload and contractility), increased wall stress or inadequate oxygen supply [Laughlin et al 2012].

The coronary vasculature responds to a wide range of stimuli: mechanical forces, neurohumoral agents, metabolic mediators or neural innervation. Parasympathetic vagal stimulation is able to dilate resistance vessels through acetylcholine release. Sympathetic stimulus is achieved during stress either directly through innervation of the heart and coronaries or through circulating catecholamines. Both epinephrine and norepinephrine stimulate adrenergic β_1 - and β_2 -receptors causing vasodilation, while β_1 stimulation also increases myocardial oxygen consumption. Vasoconstriction is caused by α_1 -receptor activation but in physiological situations this effect is abolished by the β-adrenergic and metabolic responses. Pigs usually lack coronary α-receptors. Pre- and postsynaptic α_2 -activation seems to be less significant in controlling CBF. The coronary microvasculature also responds to changes in shear stress which leads to "flow-induced" vasodilation. Nitric oxide

(NO) and endothelium-derived hyperpolarizing factor are thought to be responsible for this mechanism. Many of the vasodilatory effects are endothelium-dependent or mediated by NO. Endothelial nitric oxide synthase (eNOS) is activated by several factors such as acetylcholine, bradykinin or histamine. Other soluble factors, adenosine triphosphate (ATP), adenosine diphosphate (ADP), substance P, thrombin, serotonin and vasopressin also cause coronary vasodilation in a NO-dependent manner. ATP is in addition able to cause vasoconstriction by acting on smooth muscle cells by sympathetic activation. NO diffuses to the vascular smooth muscle cells (VSMC) where it increases the formation of cyclic guanosine monophosphate (cGMP) which leads to the opening of K^+ and Ca^{2+}-channels and VSMC relaxation. Adenosine is a potent vasodilator under hypoxic conditions. It is formed by the degradation of ATP and adenosine monophosphate (AMP) during increased energy consumption and acts primarily on the P1-receptor subtypes A_1 and A_2 [Farhad et al 2013, Duncker 2015].

2.3 Blood coagulation and thrombosis formation

The coagulation proteins are divided into enzymes (serine proteases) and cofactors. Blood coagulation is initiated after vascular injury by exposure of the transmembrane surface molecule tissue factor (TF). TF is not expressed on endothelial cells or circulating blood cells. The exposed TF binds to factor VII and is activated by circulating activated factor VII (FVIIa). The TF + FVIIa complex activates factor IX which interacts with factor VIIIa and activates factor X (FXa). FXa and activated factor V catalyzes the reaction from prothrombin to thrombin. Thrombin is a multifunctional molecule that is soluble and does not require a cofactor. Thrombin cleaves fibrinogen into fibrin and also activates factors V, VIII, XI, XIII, platelets and thrombin activatable fibrinolysis inhibitor. Thrombin exerts negative feed-back on the coagulation system by activating fibrinolysis and protein C. The cleavage of fibrinogen allows the fibrin monomers to polymerize. Activated factor XIII (FXIIIa) then stabilizes this loose clot by cross-linking the polymers. The fibrinolytic system limits thrombus formation by cleavage of fibrin and fibrinogen bonds by plasmin. Plasmin is activated from plasminogen by tissue plasminogen activator (tPA) or urokinase. tPA and urokinase are incorporated into the newly formed fibrin clot and limits clot growth protected from soluble plasminogen activator inhibitor-1 and α2-antiplasmin (Figure 1).

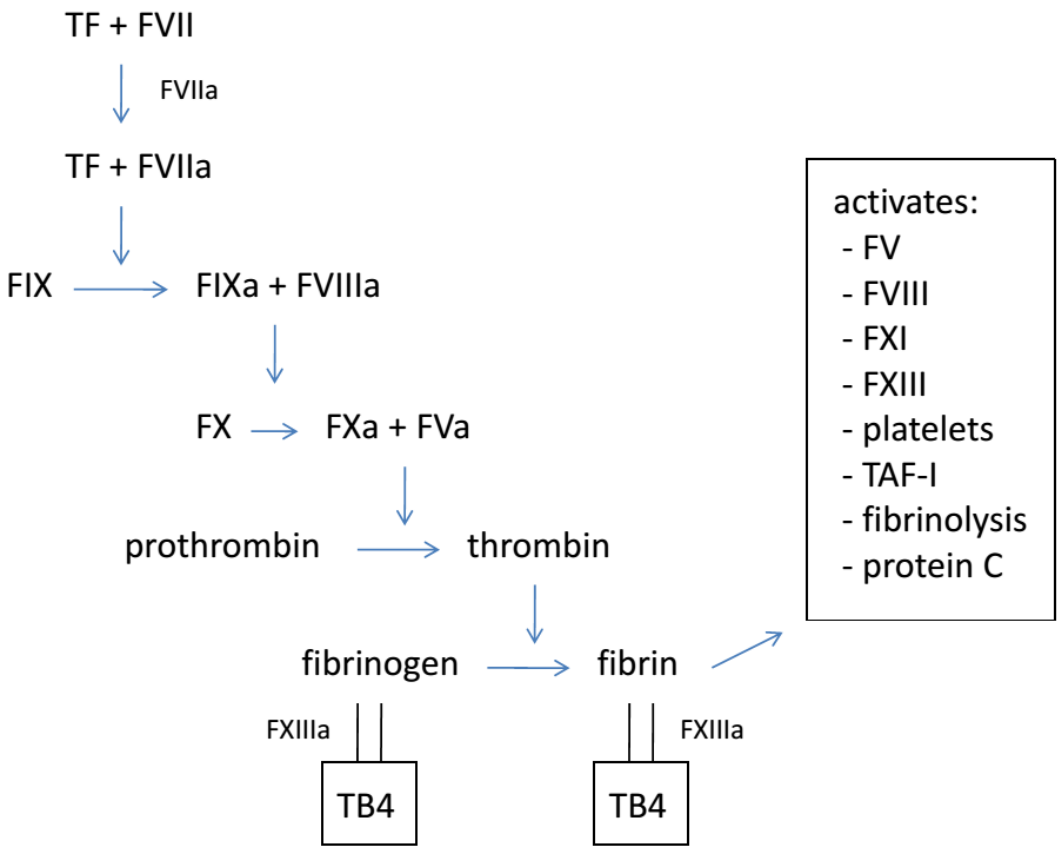

Figure 1. The coagulation cascade is initiated by tissue factor (TF) binding to factor VII (FVII) and activated by circulating activated FVII (FVIIa). Thymosin beta 4 (TB4) is covalently bound to fibrinogen and fibrin by FXIIIa.

Platelets are produced in the bone marrow from their precursors, megakaryocytes and their production is stimulated by thrombopoietin. The platelets are either freely circulating or pooled in the spleen and has a lifetime of 7-10 days. Platelets adhere to injured endothelium and aggregate with other platelets. Exposed collagen in blood vessels binds von Willebrand factor (vWF) which in turn binds to platelets glycoprotein Ib receptor and leads to platelet activation. After activation the platelets release vWF, FV, serotonin and ADP leading to further platelet activation. There is also an increase in thromboxane A_2 production. Platelets aggregate through fibrinogen after activation of the glycoprotein IIb/IIIa (GpIIb/IIIa) receptor. GpIIb/IIIa is activated by binding of vWF to GpIb, thromboxane A_2 and ADP. The most potent platelet activator is however thrombin. Activated platelets expose phosphatidylserine on their surface and increase their surface area by the production of microparticle blebs. The exposed phosphatidylserine binds coagulation factors increasing coagulation efficiency.

Coagulation is balanced with a natural anticoagulant for each reaction in the coagulation cascade. TF pathway inhibitor binds to FXa which further binds to FVIIa inhibiting activated factor IX formation. Protein C acts with its freely circulating cofactor protein S to cleave fVa and FVIIIa. Antithrombin binds to and inactivates all serine proteases of the coagulation system. Its function is markedly increased by heparan or heparin [DeLoughery T 2015].

2.4 Myocardial infarction and ischemic cardiomyopathy

2.4.1. Coronary artery disease

CAD is one of the leading causes of morbidity and mortality in the western world. The disease process usually starts during the first decades of life and remains silent until the fifth or sixth decades. The initial step in CAD development, or atherogenesis, is the incidence of endothelial injury or inflammation. There are several triggers for this inflammatory response and they are often related to the clinical risk factors of the patient such as hypertension, dyslipidemia, diabetes, smoking or genetic factors. Toxins, mechanical shear stress, pro-inflammatory cytokine signaling, glycoxidation products are some of the potential initiators of this inflammatory response which leads to increased expression of adhesion molecules on the endothelium and subsequent attraction of inflammatory cells from the circulation. The transmigration of T-lymphocytes and monocytes into the intima and the interplay between these leukocytes and the resident endothelial and smooth muscle cells maintains the inflammatory response and leads to processes responsible for intimal thickening and later atherosclerotic plaque formation. Within the plaque there are several processes taking place. Migrating smooth muscle cells are responsible for the fibromuscular remodeling of the extracellular matrix. The extracellular matrix is able to bind lipoproteins and in this inflammatory milieu the molecules are prone to oxidative modification further enhancing inflammation. Macrophages are unable to degrade the modified lipoproteins and this ultimately leads to the production of “foam” cells and cell necrosis. There are two different types of atherosclerotic plaques, stenotic and non-stenotic. Non-stenotic plaques consist of a lipid-rich core with a thin intimal cap covering the plaque. The artery is usually compensatory enlarged without narrowing of the arterial lumen. Stenotic plaques are fibrotic with a thicker fibrous cap. There is less compensatory enlargement and therefore earlier obstruction of the vessel lumen. The stability of coronary plaques is related to their composition which influence the risk for plaque rupture and thrombosis [Fuster et al 1992, Libby et al 2005, Ambrose et al 2015].

2.4.2 Myocardial infarction

MI can be the first sign of CAD or occur repeatedly. MI can pathologically be defined as myocardial cell death following ischemia. Cell death starts after an ischemic period of 20 minutes but complete necrosis can usually not be detected until several hours after the event. Several factors influence the myocardium's resistance to ischemia, such as oxygen demand, collateral circulation or cell susceptibility to ischemia. The incidence of myocardial cell death is detected by biomarker analysis. The preferred method is cardiac troponin I (cTnI) or T (cTnT) measurement as they are highly cardiac specific and clinically sensitive. Alternatively, creatine kinase MB isoform can be analyzed. The detection value for both is set as a value exceeding the 99th percentile of a reference population. Cardiac biomarkers should be analyzed at patient referral and at least once 3-6 hours later. Biomarker release can also be related to other pathology than MI, therefore risk profile analysis and other tests are needed to differentiate between MI and other causes. The manifestations of MI on an electrocardiogram can differentiate between acute ischemia and prior infarction according to several specific changes, usually ST-segment changes or the incidence of Q-waves. MI is universally classified into five types. Type 1 or spontaneous MI is related to thrombus formation or thromboembolism within the coronary circulation. Acute coronary thrombus can occur after atherosclerotic plaque rupture, erosion, fissuring or dissection. The other types are related to an imbalance between oxygen supply and demand for other reasons than CAD, procedure related MI or suspected MI resulting in death before blood sampling [Thygesen K et al 2012, Ambrose et al 2015].

2.4.3 Inflammation and fibrosis after myocardial infarction

The cellular phases after MI are divided into the inflammatory response, proliferative phase and maturation phase. After infarction there is immediate loss of endothelial integrity leading to inflammatory cell infiltration. The first inflammatory cells to invade the infarcted myocardium are neutrophils and cytotoxic T-lymphocytes. Later there is monocyte infiltration and activation of tissue resident macrophages and mast cells. Infiltration and activation of these cells leads to a rapid and strong inflammatory response. The cellular death of cardiomyocytes, endothelial cells and fibroblasts through necrosis, apoptosis or autophagy further enhances inflammation and if there is restoration of blood flow to the infarcted area I-RI will occur. After activation the inflammatory cells starts to secrete chemoattractant and pro-inflammatory cytokines. Necrotic or stressed cells release danger-associated molecular patterns (DAMPs) such as interleukin-1α (IL-1α), ATP, ribonucleic acid (RNA), heat shock proteins or the protein high mobility group box 1. These DAMPs activate toll-like and IL-1 receptors activating pro-inflammatory pathways like mitogen activated protein kinases or NF-κB. NF-κB is a key promoter of acute inflammation and upregulates TNF-α, different interleukins (IL-1β, IL-6,

IL-18) and adhesion molecules for T-lymphocytes and monocytes [Nian et al 2004, De Haan et al 2013].

The duration of this rapid inflammatory response is 3-4 days in mice and is necessary to effectively clear the necrotic cells and cellular debris from the infarct area. The proliferative phase begins by the end of the first week after MI. The initial step in this phase is the suppression of inflammation. Late stage and apoptotic neutrophils suppress inflammation by secreting anti-inflammatory cytokines like lipoxins or resolvins, annexin A1 and lactoferrin. They are also responsible for producing decoy or scavenging receptors for pro-inflammatory cytokines, leading to their depletion. Phagocytosis signals polarize macrophages in the direction of their anti-inflammatory M2 phenotype. M2 macrophages secrete anti-inflammatory and pro-fibrotic cytokines like IL-10 and TGF-β. In mice this polarization takes place at approximately day 7 after MI. There is also an influx of dendritic cells and different populations of T-cells aiding in the resolution of inflammation and the start of fibrosis and angiogenesis. Maturation of neovessels reduces capillary permeability and decreases further inflammatory cell transmigration into the injured myocardium. There is also evidence for a regulatory role of surviving border-zone cardiomyocytes in the proliferative phase.

At the end of the proliferative phase there is initiation of extracellular matrix reorganization and scar formation. The infarcted matrix is phagocytized and a provisional matrix consisting of fibrin and fibronectin is formed. This matrix serves as a scaffold for matricellular non-structural proteins and migrating and proliferating fibroblasts. The fibroblasts are activated into myofibroblasts which recellularize the infarct area. The cells originate from resident or circulating fibroblasts or epicardial progenitors but they are also thought to transdifferentiate from endothelial cells. After the scar tissue has formed the extracellular matrix matures by cross-linking of its collagen. Most of the reparative and inflammatory cells are deactivated and undergo apoptosis. The process finally ends in resolution of the proliferative phase or progresses into late remodeling of the myocardium [Frangogiannis et al 2012, Prabhu 2016].

2.4.4 Management of coronary artery disease and myocardial infarction

The key in CAD treatment is prevention and risk factor management. General prevention includes diet and exercise as well as smoking cessation. For asymptomatic patients with additional risk factors or increased risk, specific treatments such as antihypertensive, lipid lowering, and blood glucose lowering treatments should be considered. Antiplatelet therapy with aspirin is also recommended, especially in diabetic patients with increased risk. Patients with symptomatic CAD benefit from

additional anti-ischemic therapy with β-blockers and nitrates. After acute myocardial infarction immediate antithrombotic therapy is recommended together with attempts for early revascularization [Prabhu et al 2016].

2.4.5 Cardiac structural remodeling in ischemic cardiomyopathy

Ischemic cardiomyopathy is characterized by regional ventricular hypokinesia or akinesia, wall thinning and ventricle enlargement and change of shape from ellipsoid to spherical. Normally heart contractility is determined by preload according to the Frank-Starling law, with increased contractility when ventricular filling increases. Ventricular enlargement and dysfunction in the ischemic heart leads to excessive filling pressures and volume overload resulting in reduced contractility and cardiac output (calculated as stroke volume times heart rate). The ischemic heart might also contain areas of hibernating myocardium in addition to infarcted areas. Myocardial hibernation is related to prolonged or repetitive periods of ischemia and results in reduced contractility of cardiomyocytes. Especially subendocardial regions are susceptible to ischemic hibernation due to increased filling pressure and ventricular dilation. The heart tissue undergoes structural changes during heart failure development that is related to sustained inflammation after MI. This is seen as myocardial fibrosis and myocardial cell death due to apoptosis and necrosis. Large initial MI size or excessive post-infarction inflammation are factors related to increased risk for late remodeling [Cohn et al 2000, Johnson 2014, Prabhu et al 2016].

2.4.6 Neurohumoral activation

During heart failure development there is activation of the neurohumoral system. The decrease in cardiac output and vascular underfilling activates cardiovascular baroreceptors and increases sympathetic tone with an increase in vasoconstriction, heart rate and blood pressure. In chronic situations this normal vasoactive response becomes maladaptive. In the heart there is downregulation of β-adrenergic receptors and the renin-angiotensin-aldosterone (RAA) system is activated. Angiotensin II activation increases catecholamine and renin release causing further vasoconstriction while increased aldosterone activity causes sodium and fluid retention. There is also release of atrial and brain natriuretic peptides but these molecules are short-lived in the circulation. The neurohumoral activation leads to fluid retention, impaired cardiovascular performance and further progression of cardiomyopathy [Braunwald 2013, Johnson 2014].

2.4.7 Management of ischemic heart failure

Heart failure is managed with medications suppressing RAA activity and β-blockers. Fluid retention often requires diuretic therapy. In advanced heart failure neprilysin inhibitors, may be used. This agent inhibits the degradation of vasoactive peptides like natriuretic peptides or bradykinin and leads to vasodilation and natriuresis. Cardiac performance can also be improved with cardiac resynchronization therapy. Any associated pathology causing further cardiac damage such as ischemia or valve disease should be addressed and if possible treated with revascularization or valve procedures. In end-stage heart failure inotropic therapy might be needed. At this stage definitive therapy with ventricular assist device implantation or heart transplantation should be considered. Gene and stem cell therapies are so far experimental but might become an option for selected patients in the future [Bernstein et al 2012, Braunwald 2013, Johnson 2014].

2.5 Pathogenesis of acute and chronic anthracycline-induced cardiomyopathy

Doxorubicin is a widely used anti-cancer drug that belongs to the anthracycline family. Its use is associated with cardiotoxicity and doxorubicin-induced cardiomyopathy animal models are commonly used in heart failure research. Doxorubicin cardiotoxicity can be divided into acute and chronic forms. The acute form decreases cardiac function within the first week of use and is reversible with discontinuation of the drug. The chronic forms develop early within a year, or late more than a year after treatment and lead to dilated cardiomyopathy. Doxorubicin binds to DNA and inhibits DNA and RNA synthesis. It also inhibits the function of topoisomerase II causing cessation of mitotic activity in cancer cells but also in normal cell types. The cardiotoxic effects are related to free radical production and lipid peroxidation of cellular membranes. Other cardiotoxic effects are secondary to decreased ATP production, mitochondrial damage, apoptosis, reduced DNA synthesis and impaired protein function. Acutely this can be observed as release of cardiac biomarkers and a transient decrease in cardiac contractility. The chronic changes manifest as reduced contractility, dilatation and fibrosis of the ventricles. In pediatric patients a restrictive phenotype of the cardiomyopathy can be seen which is related to more pronounced fibrosis. The cardiotoxic effects are dose and time dependent. Cumulative doses exceeding 500 mg/m^2 and high single dose administration are associated with increased risk. Concomitant chemo- or radiation therapy or pre-existing co-morbidities are other important risk factors. Pegylated and liposomal formulations of doxorubicin (PLD) are considered to be safer with respect to cardiotoxicity. In these formulations the drug is carried in a lipophilic carrier vesicle with polyethylene glycol (PEG) attached to its surface. The carrier prevents opsonization and clearance of the molecule in the reticuloendothelial system and also reduces the excretion rate in bile and urine. This leads to longer pharmacokinetic action of the drug and more blunted peak concentrations after admin-

istration. PLD enters tissues with increased permeability such as tumors with active neovascularization while normal or intact tissues are fairly resistant to its extravasation. PLD has clinically been shown to be safe with cumulative doses exceeding 500 mg/m^2 and the maximal tolerated single dose is 60 mg/m^2 every four weeks. PLD causes mainly epithelial toxicity on skin or mucosa of the gastrointestinal tract. Mild myelosuppression and nausea are other adverse reactions. Acute infusion reactions are considered to be related to the liposomal coating and not the drug itself and are usually self-limiting. PLD is considered relatively non-cardiotoxic in clinical situations. A dilated-type cardiomyopathy has however been observed in a few experimental studies on mice with high single doses [Solomon et al 2008, Raj et al 2014].

2.6 Cardiac surgery and myocardial ischemia-reperfusion injury

2.6.1 Surgical technique and myocardial preservation methods

The use of CPB is mandatory in many cardiac surgery procedures. CPB allows the surgeon to carry out the procedure in a quiet and bloodless operating field and also prevents air embolism into the systemic circulation. During CPB, the distal ascending aorta is usually cannulated for systemic perfusion but other peripheral arterial routes are also possible such as the axillary or femoral arteries. Venous drainage with a sequential cannula through the right atrial appendage is most commonly used, other methods are bi-caval or femoral vein approaches. After the arterial and venous lines are connected extracorporeal perfusion with a heart-lung machine can be started. With extracorporeal circulation and blood gas exchange in the heart-lung machine's oxygenator the aorta can be cross-clamped with the heart and lungs isolated from the circulation. After aortic cross-clamping there is cessation of myocardial blood flow.

Myocardial preservation or protection can be achieved by infusing different types of cardioplegic solution either into the proximal aorta in an antegrade fashion or through the coronary sinus by retrograde infusion. The cardioplegic solution contains potassium causing electrochemical arrest of the heart. This obviously reduces cardiac oxygen consumption but other methods to further reduce myocardial oxygen consumption are often applied such as the use of hypothermic cardioplegia or topical cooling of the heart. There are numerous types of cardioplegia in routine use. In addition to potassium most types contain different buffers, energy substrates and electrolyte supplements. Cardioplegia can be used as such when it is referred to as crystalloid cardioplegia or be mixed with blood, and can be delivered as warm, tepid or cold infusions. Myocardial oxygen demand can be reduced further by optimizing the cardiovascular system pre-operatively or by mild systemic hypothermia during the procedure [Bolli et al 2004, Hausenloy et al 2012].

2.6.2 Systemic inflammatory response

Cardiopulmonary bypass induces a strong systemic inflammatory response syndrome (SIRS) that is related to hemodynamic instability and increased morbidity and mortality in patients. CPB causes inflammation due to blood contact with foreign surfaces, ischemia-reperfusion injury of end-organs and by endotoxemia related to splanchnic hypoperfusion. Several studies have compared coronary artery bypass grafting (CABG) patients undergoing procedures with or without CPB-assist. In these studies, CPB was related to increased neutrophil activation, endothelial adhesion molecule expression and complement activation. The cytokine profiles in CPB-assisted procedures showed increased concentrations of pro-inflammatory cytokines like IL1, IL6, IL8 and TNF-α. There are several biomaterial dependent and independent factors influencing this response. The type of circuit, blood pump or oxygenator used are factors that can be modified by shortening the circuit, using heparin-coated or more biocompatible tubing or components causing less mechanical stress on blood cells. The patient's comorbidities and perioperative hemodynamics are important factors that should be optimized before surgery. Surgical factors like the amount of tissue trauma, blood loss and duration of the procedure are likely to aggravate SIRS. Perioperative and post-operative anesthetic considerations include factors like choice of anesthetic agents, optimization of perfusion conditions and temperature as well as minimizing the duration of mechanical ventilation and preventing kidney injury and risk for renal replacement therapy. Different pharmacological approaches for reducing CPB-induced SIRS have been proposed. The use of anti-inflammatory substances such as aprotinin, pentoxifyllin or corticosteroids might limit inflammation. Free radical scavengers or antioxidants and inhibitors of endothelial cell activation are theoretically beneficial and have shown some effect in experimental settings [Suleiman et al 2008, Millar et al 2016].

2.6.3 Myocardial ischemia-reperfusion injury

Cardiac I-RI is caused by ischemia and restoration of myocardial blood flow after the ischemic insult. Coronary artery obstruction or aortic cross-clamping in surgical situations leads to ischemia and revascularization or de-clamping of the aorta to reperfusion injury causing reversible or irreversible myocardial injury. Reversible injury triggers arrhythmia and temporary loss in contractility or myocardial stunning while irreversible damage is associated with cardiomyocyte death starting within minutes of reperfusion [Kloner et al 2001]. There are several mechanisms responsible for I-RI. Cellular depletion of ATP impairs the function of cell membrane transport of Na^+ and Ca^{2+} by Na^+-ATPase leading to intracellular calcium overload. Intracellular acidosis is caused by anaerobic metabolism and decreased metabolite washout. With restoration of blood flow there is generation of reactive

oxygen species (ROS) and additional calcium overload of cardiomyocytes. Calcium overload is associated with arrhythmia and hyper-contracture of cardiomyocytes. ATP depletion, calcium overload and ROS cause damage to mitochondrial membranes and increases their permeability and also increases opening of mitochondrial transition pores leading to the entry of mitochondrial proteins into the cytosol which trigger cardiac cell death. A no-reflow phenomenon can cause further ischemic damage to the myocardium. This is caused by injury and swelling of endothelial and myocardial cells or vasospasm causing microvessel obstruction. The phenomenon can also be explained with the formation of microthrombi and leukocyte plugs [Boyle et al 1996, Verrier et al 1998]. In experimental and some clinical studies, I-RI has been attenuated by ischemic pre- or postconditioning. This is a method where the organ at risk is subjected to several short non-lethal episodes of ischemia with short reperfusion periods in between. This can be performed either before or after the onset of index ischemia and can also be performed remotely in another organ for preconditioning. These methods are associated with the activation of prosurvival signaling pathways. The reperfusion injury salvage kinase (RISK) pathway phosphorylates and activates the proteins Akt, PI3K, ERK1/2 and MEK1/2. These proteins cause further downstream signaling inhibiting mitochondrial dysfunction, apoptosis and reducing calcium overload. The survival activating factor enhancement (SAFE) pathway is activated by TNF-α and other cytokines and involves upregulation of the cardioprotective proteins NF-κB, iNOS, COX-2 and heat shock proteins. NF-κB seems to have a dual role as both a pro- and anti-inflammatory mediator [Anselmi et al 2004, Raja et al 2005, Hausenloy et al 2016].

2.7 Cardiac imaging in ischemic and non-ischemic heart disease

2.7.1 Echocardiography

Echocardiography is non-invasive and its use is fairly inexpensive. It is however user and also patient dependent with some inter- and intraobserver variability in analysis outcome. Echocardiography is a good tool for assessing sizes and volumes of the heart chambers, systolic and diastolic function, intracardiac pressures and valvular function. In certain circumstances there can however be underestimation of ventricular volumes and ejection fraction but this can be overcome with the use of intravascular contrast agents like microspheres. In ischemic cardiomyopathy segmental wall thinning and wall motion abnormalities of the ventricles is usually observed with patterns corresponding to the coronary circulation. An end-diastolic wall thickness of less than 6 mm in the LV is considered equal to infarction and scar formation. In non-ischemic heart disease, the pattern is more diffusely distributed with more common involvement of the RV. With the use of pharmacological

or physiological stress the ventricles can be assessed for inotropic reserve. Ischemic and hibernating myocardium retains its contractility reserve while scar tissue does not. The use of contrast agents is sometimes used for determining myocardial perfusion [Saskia et al 2007, Ananthasubramaniam et al 2011].

2.7.2 Cardiac magnetic resonance imaging

Cardiac magnetic resonance (CMR) imaging is better for assessing the same parameters as with echocardiography except for diastolic function. CMR is more time-consuming, expensive and equipment-dependent but offers superior spatial resolution and is more accurate and reproducible. Myocardial viability can be evaluated by using perfusion sequences and gadolinium contrast agent during rest or stress. In normal myocardium there is fairly quick washout of the agent while ischemic areas in the heart show delayed clearance. The resolution allows for precise anatomical location of hypoperfused segments and can discriminate between subendocardial or transmural ischemia. In non-ischemic cardiomyopathies and after MI, CMR can show signs of myocardial edema. Local or diffuse contrast enhancement is generally a sign of myocardial fibrosis caused by chronic MI and can be used to estimate the amount of myocardial damage [Viswamitra et al 2004, Koskenvuo et al 2007, Saskia et al 2007, Ananthasubramaniam et al 2011].

2.7.3 Positron emission tomography

Myocardial perfusion can be quantified by positron emission tomography (PET) with the use of several tracers. Radiolabeled water (^{15}O-H2O) diffuses freely across cell membranes and its distribution is independent of metabolic factors. Its use is well validated in assessing myocardial blood flow during rest and pharmacological stress [Knuuti et al 2009].

2.8 Experimental animal models for myocardial infarction, anthracycline-induced cardiomyopathy and ischemia-reperfusion injury

Experimental animal models for myocardial ischemia and ischemia-reperfusion injury are crucial in the translation of novel therapeutics into clinical phase trials. In basic cardiovascular research the most commonly used animal is mouse. In the human genome there are murine orthologs for 99 % of the genes. There are also numerous genetically modified mouse strains available for research in specific areas of cardiovascular biology. Mouse models are inexpensive and housing of the animals is simple. The animals fast breeding rate and short life span are factors suitable for high-throughput studies. There are however several limiting cardiac differences between mice and larger mammals or humans. Heart contractility, hemodynamics, pathophysiology of cardiac disease and differences in pharmaceutic

response are important factors that need to be considered. As mentioned previously pigs are very similar to humans in cardiac function and pathophysiology. Housing and costs are however some of the limiting factors related to their use in research. Pigs are also prone to ischemia-induced arrhythmia which increases mortality. The failure to translate positive preclinical findings in cardioprotection research is related to several issues. Animal studies usually consist of otherwise healthy and rather homogenous populations, which increases the risk for a type 1 error or false positive findings. The lack of comorbidities in animals and the differences in pharmacological study environment are associated with poor translation of protective therapeutics. A recent workshop recommendation stated some key points related to better translation of preclinical research. It stated that efficacy of novel therapies should be demonstrated in large animal models preferably with induced cardiovascular comorbidities in a reproducible manner. Studies should also address the polypharmaceutical environment and mimic clinical situations. It was also recommended that surrogate end-points should be accompanied with hard end-points such as mortality [Bell et al 2016].

The most commonly used myocardial infarction model is the mouse LAD ligation model. The technical success of this procedure is highly user-dependent. The ligature is placed below the left atrial appendage and occlusion is observed as pallor of the myocardium distally to the ligation. Standardizing infarct size with this procedure is difficult. Ligation models in pigs are more challenging as proximal ligation of the main coronaries usually results in fatal arrhythmias. Better success can be achieved with ameroid constrictors or so called “bottle-neck” stents causing gradual occlusion of the coronaries. These models cause fairly standard-sized infarcts with reasonable mortality but are time-consuming and expensive. With MI models there is also infarct-size and time-dependent remodeling of the LV leading to ischemic heart failure [Rissanen et al 2013, Tarkia et al 2014, Camacho et al 2016].

Anthracycline-induced cardiomyopathy is widely used as an animal model. In this model mice are administered single or repetitive doses of the anthracycline drug doxorubicin which causes either acute or chronic myocardial injury leading to a dilated cardiomyopathy phenotype. The cardiotoxicity is related to free radical production with damage to mitochondria, peroxidation of cellular membranes and upregulation of inflammatory cytokines [Zhu et al 2008, Farhad et al 2016, Pecoraro et al 2016].

There are several different pig models for cardiac ischemia-reperfusion injury. Closed-chest models rely on percutaneous balloon-occlusion of single coronary arteries. Surgical models can also be used for external temporary ligation of coro-

nary vessels. In cardiopulmonary bypass-assisted models global myocardial ischemia-reperfusion injury is induced by clamping and de-clamping of the aorta. In these models the heart is also protected from ischemia with the use of cardioplegia. Most I-RI studies performed on pigs have been acute experiments with follow-up periods of hours up to a few days. Infarct size, biomarkers for cardiomyocyte death or apoptosis, and functional parameters are the most commonly used end-point parameters in these studies. Unprotected local ischemia-reperfusion injury causes substantially more cardiac damage than protected global I-RI [Malmberg et al 2011]. Modern preservation techniques in CPB assisted cardiac surgery are very effective and the use of this model in cardioprotection research has been criticized because benefit is difficult to demonstrate with its very limited therapeutic window. Prolonging ischemia is one method to overcome this limitation but excessive ischemic insult usually results in increased mortality and decreased efficacy of therapies. In addition to the issues mentioned previously there is one significant hurdle in translating the benefits of novel therapeutics into clinical practice. Dose-response curves are often poorly known for both pharmacological and methodological protective strategies making the determination of conditioning or protection thresholds impossible [Bell et al 2016].

2.9 Thymosin beta 4

TB4 is a 43 amino acid peptide originally isolated from thymus extracts. TB4 is the most abundant of three different beta thymosins in human, the others are thymosin beta 10 and 15. The polypeptide is water soluble and acidic with a molecular weight of 4,964 kDa [Huff et al 2011]. It has been found in all studied cell types except for erythrocytes. High concentrations are found in platelets, leukocytes and wound fluids. TB4 does not bind to heparin and is therefore not considered a growth factor [Hannappel et al 1987]. The peptide is found in the cytosol or nucleus of cells and it lacks a signal sequence for excretion [Watts et al 1990]. It is unknown if there is an active excretion mechanism for the peptide or if it is only released through passive translocation or after cell membrane damage. Serum levels of the peptide range from 0.005- 0.01 µM, but in inflammatory conditions concentrations of 0.1- 600 µM have been reported [Bicer et al 2011, Song et al 2012]. Due to its chemical properties the peptide is rapidly cleared from the circulation and internalized by endothelial and other cells. After exogenous administration peak concentrations in serum is achieved within minutes and in the heart after 2 hours. TB4 is the sole precursor of the antifibrotic and proangiogenic peptide Ac-SDKP, which is degraded by angiotensin converting enzyme (ACE) [Mora et al 1997, Kaur et al 2012].

TB4 is the main intracellular G-actin sequestering peptide and regulates actin polymerization. The peptide is cross-linked to actin, collagen, fibrin and fibrinogen

by factor XIIIa and also influences platelet adhesion rate. At lower concentrations platelet deposition is attenuated and with increasing dose the rate returns to normal [Huff et al 2002]. The peptide has interactions with several cellular proteins. TB4 increases formation of the PINCH1-ILK-α-parvin (PIP) complex. After its formation the PIP complex localizes to the membrane bound focal adhesion complex and regulates cell shape, motility and survival. The prosurvival downstream signaling molecule Akt (protein kinase B) is phosphorylated in a ILK-PINCH-1 or PI3K dependent manner and this is increased by TB4. The peptide is also related to upregulation of VEGF and HGF. There seems to be a dual interaction between the latter as HGF is also able to upregulate TB4. TB4 acts as an anti-inflammatory mediator by reducing TNF-α mediated NF-κB activation and by decreasing IL-6 signaling. TB4 is also related to reduced reactive oxygen species generation by upregulation of anti-oxidant enzymes. In cardiac fibroblasts it upregulates catalase, Cu/Zn SOD and the antiapoptotic molecule Bcl2 while downregulating the proapoptotic signals Bax and caspase-3 [Wei et al 2012]. The peptide also influences connective tissue production by downregulating connective tissue growth factor, collagen 1 and collagen 3. Recently it was discovered that TB4 is able to decrease angiotensin II mediated cardiomyocyte growth by blocking β-catenin translocation into the nucleus. Of extracellular receptors, TB4 has been shown to bind to cell surface ATP synthase promoting ATP production and stimulate endothelial cell migration (Figure 2.) [Bock-Marquette et al 2004, Freeman et al 2011].

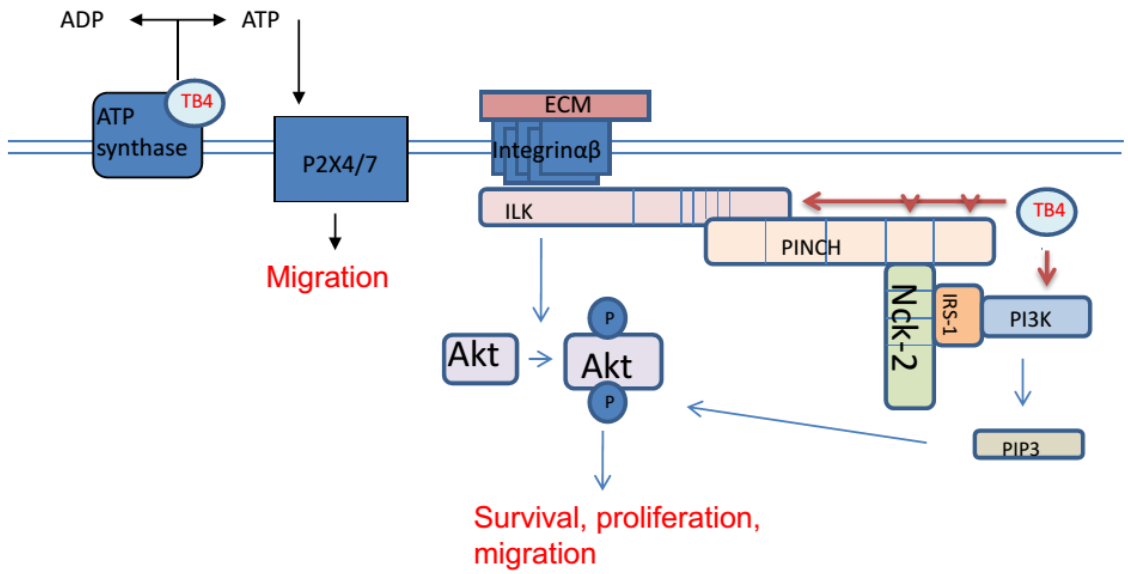

Figure 2. The identified cellular binding sites of thymosin beta 4 (TB4). TB4 binds to the ILK-PINCH complex or interacts with phosphoinositol 3-kinase (PI3K) and increases Akt phosphorylation. This activates downstream signaling routes responsible for cell survival, proliferation and migration. TB4 also binds to cell surface F1F0 ATP synthase and is able to induce endothelial cell migration through purinergic signaling.

By the cellular mechanisms described previously or by still unknown targets of the peptide, TB4 is able to impact cell and tissue behavior in several ways. In neoplasms, upregulation of TB4 is associated with increased tumor growth, metastasis and angiogenesis. In different in vitro and in vivo experiments the peptide has several protective or reparative functions. It increases cell survival in cardiomyocytes by reducing apoptosis and necrosis likely by Akt-mediated signaling and induces angiogenesis by promoting endothelial cell mobilization and migration. The anti-inflammatory effects are related to decreased TNF-α and NF-κB signaling and downregulation of IL-6. TB4 also inhibits leukocyte and macrophage transmigration into injured tissues. Free oxygen radical production at sites of tissue injury is counteracted by TB4-mediated upregulation of anti-oxidative enzymes in cardiac fibroblasts. Free radicals also cause TB4 sulfoxidation and this modulated peptide has been shown to induce transmigration of macrophages. The influence on cardiac healing after MI seems to be dual in nature. TB4 is known to promote extracellular matrix synthesis but on the other hand it reduces excessive fibrosis. Cardiac repair has been linked to TB4-induced activation of epicardium-derived progenitor cells and their migration into the damaged myocardium. The fate of these cells is somewhat unclear but enhancing this process could be one key in regenerating lost myocardium after infarction [Zhou et al 2011, Kispert et al 2011, Smart et al 2011, Goldstein et al 2012, Gupta et al 2012, Bollini et al 2015, Li et al 2016].

2.10 **Purinergic signaling**

The extracellular signaling of ATP, ADP and adenosine through purinergic receptors is named purinergic signaling. The high-energy triphosphate ATP is released from cells after mechanical stimuli or cell membrane rupture but can also be secreted by activated inflammatory cells. ATP is hydrolyzed by cell-surface or soluble nucleotidase enzymes to ADP and AMP. AMP is further degraded to adenosine by the enzymes alkaline phosphatase and 5'-endonucleotidase (CD73). ATP and ADP are strong danger signals and act as a chemoattractants to circulating leukocytes at sites of tissue injury. They also regulate vascular tone and platelet aggregation. In normal circumstances vasomotor tone is controlled by actions on different subtypes of P2X or P2Y receptors on endothelium or smooth muscle in the vessel wall causing vasodilation or vasoconstriction. During tissue injury the increase in ATP causes mainly vasoconstrictor effects by activation of smooth muscle receptors. In the heart ATP increases inotropy, but can also provoke arrhythmia. ADP induces platelet aggregation through the P2Y12-receptor. During tissue damage the amount of available ATP and adenosine is balanced by the rate limiting enzyme CD73, which after injury mainly is derived from leukocytes. Adenosine binds to P1-receptors and leads to pro- or anti-inflammatory signaling activation depending on the receptor subtype. After myocardial injury adenosine is cardioprotective by limiting the inflammatory response, decreasing fibrosis and

improving scar maturation [Eckle et al 2007, Bönner et al 2013, Jalkanen et al 2015].

2.11 Chitinase 3-like-1

Chitin is the second most abundant polysaccharide in nature, second only to cellulose. Chitin is a structural element of the cell wall, exoskeleton and digestive system in several organisms but is absent in mammals. Chitin is degraded by true chitinase enzymes. Chitinase like proteins (CLP) are found also in mammals and are able to bind chitin without chitinolytic enzyme activity. Chitinase 3-like-1 (ch3l1) or breast regression protein 39 is a 39 kDa CLP consisting of 381 amino acids and was first discovered in mouse breast cancer cell lines but it is also present in humans. The protein is found in neutrophils, chondrocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, hepatic stellate cells and epithelial cells in the gastrointestinal or respiratory tracts. Monocytes and early macrophages are negative for ch3l1 but further differentiation of macrophages is associated with its expression. Ch3l1 expression is regulated by IL-6, IL-13, IFN-γ, vasopressin and parathyroid hormone. In chondrocytes TNF-α and IL-1β stimulate ch3l1 expression while ch3l1 inhibits the cellular responses of the cytokines in a negative feedback fashion. In ch3l1 null mice Th2-mediated inflammation is reduced indicating a role for ch3l1 in adaptive immunity. The pro-inflammatory effects of ch3l1 are also mediated by reduced apoptosis of T-cells, macrophages and eosinophils. The reduction in immune cell apoptosis and Th2-activation induces alternative (M2) macrophage activation and reparative fibrosis. By binding to cell surface syndecan-1, ch3l1 activates the focal adhesion kinase in the presence of integrin αvβ3. This initiates downstream activation of Akt and Erk which are end-stage transmitters for increased cell migration, adhesion, proliferation and cell survival [Görgens et al 2016] (Figure 3). In experimental settings ch3l1 has been shown to reduce oxidant lung injury through unknown mechanisms. In tumor biology ch3l1 upregulation is related to increased angiogenesis and metastasis [Lee et al 2011]. Ch3l1 binds to PAR2-receptors, which are expressed on cardiomyocytes but also on inflammatory cells. Pharmacological PAR2 activation offers cardioprotection after cardiac ischemia-reperfusion injury by reducing reactive oxygen species and inflammation [Antoniak et al 2011].

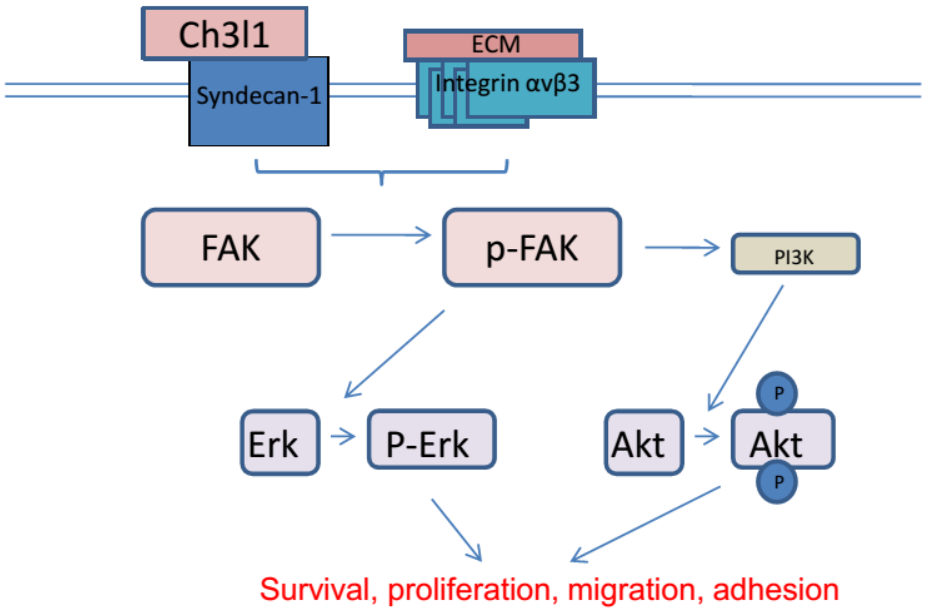

Figure 3. Chitinase 3-like-1 binds to syndecan-1 receptor which activates the focal adhesion kinase (FAK). This activation leads to the phosphorylation of Erk and Akt in a PI3K dependent manner. These signaling routes are responsible for cell signaling related to survival, proliferation, migration and adhesion.

3 AIMS OF THE STUDY

The purpose of this study was to investigate the effect of TB4 therapy after experimental myocardial injury utilizing different imaging modalities for primary outcome measurements.

The specific aims of the studies were to:

1. determine the impact of TB4 treatment on cardiac function, remodeling and blood flow in experimental animal models for MI, PLD-induced cardiomyopathy and global myocardial I-RI.

2. identify new pathways for TB4-mediated cardioprotection after MI by tissue micro-array analysis.

3. investigate the involvement of purinergic signaling in TB4 treated mice after MI.

4. determine cardioprotection of TB4 by analyzing markers for cardiomyocyte cell death and cardiac inflammation.

4 MATERIALS AND METHODS

The animal experiments were performed on male FVB/n mice and male landrace pigs. Animals were housed in individual cages with a 12:12 hour dark-light cycle and had ad libitum access to water. Mice also had ad libitum access to food pellets. At the end of follow-up, euthanasia of the mice was performed by cervical dislocation after CO2 asphyxiation. Pigs were euthanized by potassium chloride injection under general anesthesia. All animal experiments were approved by the Laboratory Animal Care & Use Committee of the State Provincial Office of Southern Finland.

4.1 Mouse myocardial infarction model (I)

Sixty-nine FVB/n mice underwent surgical ligation of the LAD and were divided into long-term and short-term study groups. For the long-term study 45 animals were divided into three groups and followed for 4 weeks. The first group (n=16) received daily intraperitoneal injections of TB4 (150 µg) (Genway Biotech, San Diego, CA, USA) for 14 days, with the first injection given 1 hour following the procedure. The second treatment group (n=17) received three doses of TB4 (400ng) at 2, 7 and 14 days post-MI injected under echocardiographic control into the peri-infarct area of the anterior left ventricular wall. The control group (n=12) received daily intraperitoneal injections of saline for 14 days post-MI. The remaining 24 animals were included into the short-term study and divided into TB4 treatment groups (n=5/time point) and control groups (n=3/time point) and followed for 2, 5 and 7 days after MI. TB4 was administered intraperitoneally 1 hour after the procedure and then daily (150 µg). The control animals received equal volumes of plain phosphate buffered saline (PBS).

4.2 Mouse PLD-induced cardiomyopathy model (II)

For the study 42 FVB/n mice were used. The animals were divided into three groups with different dosing protocols and follow-up times. Group 1 received a single intraperitoneal (i.p.) injection of PLD (Caelyx 2 mg/ml, Janssen-Cilag OY, Espoo, Finland). TB4 (150 µg) (Genway Biotech, San Diego, CA, USA) was administered i.p. daily for 14 days (n=5). Control animals received plain PBS (n=5). The animals in group 2 received a single i.p. injection of PLD and the treatment group received five doses of TB4 (150 µg) i.p. every third day (n=5). Plain PBS was administered to controls (n=5). In group 3 the animals were given four weekly i.p. injections of PLD. TB4 was given daily (150 µg) for 28 days by i.p. injection to treated animals (n=11), while control animals received plain PBS (n=11).

4.3 Pig ischemia-reperfusion injury model (III)

Ten landrace pigs were operated on and divided into a TB4 (RegeneRx Biopharmaceuticals Inc, Maryland, USA) treatment group (n=6) and a control group (n=4). A right-sided thoracotomy was performed and the aorta and right atrial appendage prepared for cannulation. After full heparinization the animals were connected to a heart lung-machine for cardiopulmonary bypass. The aorta was clamped for 60 minutes and the heart protected with antegrade cold blood-cardioplegia. TB4 was administered intravenously 2 hours before ischemia and 6 hours later (6 mg/kg). After cross-clamping the pigs were weaned from CPB and followed for 30 hours while kept sedated and on mechanical ventilation.

4.4 Echocardiography (I and II)

In study I the animals underwent echocardiography at 2 and 28 days post-MI. Echocardiography was performed using a high-frequency small animal imaging platform (Vevo 2100, Fujifilm VisualSonics Inc, Toronto, Ontario, Canada). Parasternal longitudinal images at the level of the left ventricular outflow tract were used for measuring left ventricular dimensions and ejection fraction (EF). Myocardial infarct size was determined by measuring the longitudinal length ratio of the akinetic myocardium with a wall thickness less than 50% of the normal compared to longitudinal extent of the whole LV. Early and late measurements were compared for determining progression of LV remodeling and heart failure. In study II the animals underwent echocardiography 10, 20 and 35 days after the first PLD injection. Parasternal long-axis images and M-mode images were obtained and analyzed for measuring LV fractional shortening (FS) as well as systolic (LVIDs) and diastolic internal diameters (LVIDd).

4.5 Cardiac Positron Emission Tomography (III)

Pigs underwent cardiac PET scans 25-26 hours after reperfusion to determine myocardial blood flow during rest and adenosine stress. PET studies were performed with ECAT EXACT HR+ scanner (Siemens-CTI, Knoxville, TN, USA). Myocardial perfusion was evaluated by PET with 15O-radiolabeled water both at rest and during adenosine stress. The acquisition frames were as follows: 14 × 5 s, 3 × 10 s, 3 × 20 s and 4 × 30 s (total duration 4 min 40 sec). The acquired PET data were reconstructed in 2D mode with an iterative reconstruction algorithm. Global myocardial perfusion (ml/g/min) was measured using Carimas 2 software (Turku PET Centre, Turku, Finland; http://www.turkupetcentre.fi/carimas).

4.6 Cardiac Magnetic Resonance Imaging (III)

Pigs underwent cardiac magnetic resonance imaging 27-30 hours after reperfusion. MRI studies were performed with a 1.5 T system (Philips GyroscanIntera Nova Dual MR, Philips Medical Systems, Best, The Netherlands) with a phased-array torso coil and a vector cardiographic method for ECG-gating. All acquisitions were

obtained during short breath hold periods by stopping ventilator for 5-15 seconds at a time. Each MRI study consisted of cine imaging of both ventricles for volumetrics and wall motion at rest. Late enhancement (LE) imaging was applied for scar assessment.

4.7 Histology and immunohistochemistry (I, II and III)

In the pig study (III) transmural tissue samples from the left and right ventricles were collected. Mouse hearts (I and II) were excised and cut in half along their long-axis. The samples were fixed in formalin, paraffin-embedded and then transferred to glass slides for staining. Hematoxylin-eosin and vanGieson staining was performed for general histological analysis and detection of fibrosis. Cardiomyocyte apoptosis was determined by terminal transferase nick end- labeling assay (TUNEL). Immunohistochemical staining was performed after antigen retrieval. The primary antibodies used in study I were rabbit polyclonal anti-Ki-67 (1:3000, clone AB9260, Millipore), rabbit polyclonal anti-CD68 (1:100, cat. No. bs-0649R, Bioss antibodies) and rabbit polyclonal anti-chitinase 3-like-1 (1:5000, cat. No. bs-1093R-A350, Bioss antibodies). The primary antibodies were detected with poly-HRP anti-rabbit IgG (1:1000, Bright Vision). Positive cells were counted over an ocular grid. ImageJ software was used for fibrosis quantification.

4.8 Microarray analysis and qRT-PCR (I)

Heart tissues samples from three treated and three control mice at 2 days post-MI were processed for microarray analysis with Illumina Mouse WG-6 v2.0 to detect differentially expressed genes. Expression levels of ch3l1 was analyzed on samples from 3 animals in both groups 2 and 7 days after MI. Real time quantitative (qRT-PCR) mRNA expression levels were measured with Reverse Transcriptase Core Kit (Eurogentec, cat. No. RT-RTCK-05) for cDNA synthesis and SYBR Green Master Mix (Eurogentec, Mesa Green qPCR Master Mix Plus for SYBR assay, cat. no. RT-SY2X-06+WOU) for PCR reaction. Samples were measured with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, CA, USA). Results were normalized against the expression levels of two housekeeping genes, β-actin and ribosomal S18, using CFX Manager™ software. Primer sequences for each gene were: CHI3L1(forw) 5´-GCA CAC CTC TAC TGA AGC CA-3´(rev) 5´-GCT GGT GAA GTA GCA GAC CA-3´: ACTB(forw) 5´-GCA AGC AGG AGT ACG ATG AG-3´ (rev) 5´- TAA CAG TCC GCC TAG AAG CA-3´: RSB18(forw) GAT GGG AAG TAC AGC CAG GT-3´ (rev) TTT CTT CAG CCT CTC CAG GT-3´. All primers were from Oligomer (Oligomer Oy, Helsinki, Finland).

4.9 Plasma ATP, ADP and soluble purine-converting enzymes (I)

Plasma levels of circulating ATP, ADP and soluble purine-converting enzymes were measured from TB4 treated and control animals at 2, 5 and 7 days post-MI. For quantifying plasma ATP and ADP concentrations ATPlite assay kit (Perkin Elmer) was used. For analysis of soluble, purine-converting enzyme activities samples were analyzed after optimization for each enzyme separately: (1) for ATPase, plasma suspension was incubated for 40 min with 300 μM [2,8-3H]ATP (American Radiolabelled Chemicals, St. Louis, MO) as appropriate substrate; (2) ADPase was assayed with 250 μM [2,8-3H]ADP (Perkin Elmer, Boston, MA) and incubated for 50 min with 10 μM P1,P5-Di(Adenosine-5′)Pentaphosphate (Ap5A, Sigma) to inhibit backward adenylate kinase (AK) activity; (3) ecto-5′-nucleotidase (CD73) activity was determined after 40-min incubation with 250μM [2-3H]AMP (Amersham, UK); (2) AK and NDP kinase (NDPK) were assayed with 450 μM [3H]AMP for 30 min or with 1000 μM [3H]ADP for 10 min as respective phosphorus acceptors in the presence of 800-2000μMγ-phosphate-donating ATP. 3H-labeled nucleotides and nucleosides were separated by thin-layer chromatography and then quantified by scintillation β-counting.

4.10 Thymosin beta 4 EIA and cTnT measurement (III)

Serial serum samples were collected from two pigs in both groups for pharmacokinetic studies of TB4 by enzymatic immunoassay. cTnT release was measured from plasma samples at 6 and 24 hours, post-reperfusion by electro-chemiluminescence immunoassay.

4.11 Artery wire myography (III)

Representative segments of small mesentery arteries and coronary arteries (250-350 μm in diameter) from 4 blood-donor pigs were studied by wire myography for contractile responses to TB4 in vitro. Samples were excised rapidly and placed in ice-cold oxygenated Krebs solution. Arterial rings (2 mm in length) were mounted using 40 μm wires in microvessel myograph (Danish Myograph Technologies, Aarhus, Denmark) for isometric tension recordings. After mounting, vessels were equilibrated, normalized and contracted repeatedly with 62 mM potassium chloride until maximal and reproducible contractions were obtained. To examine the effects of TB4 on mesenteric arteries concentration-response curves to TB4 (10-8 - 10-2 M) were constructed. To increase the sensitivity and to evaluate the role of NO on the effects of TB4, experiments were repeated in mesenteric arteries incubated with L-NNA (NO synthase inhibitor). Endothelium-dependent relaxation was studied in thromboxane A2 precontracted arterial rings. Data was collected and analyzed using Powerlab and Chart5 softwares (ADI Instruments, Colorado Springs, CO, USA).

4.12 Thromboelastometry Analysis (III)

Rotem® thromboelastometry (Tem Innovations GmbH, Basel, Switzerland) analysis was performed in vitro on blood samples from three pigs. TB4 was added to citrated whole blood to achieve two different concentrations (0,1 mM and 1 mM). Untreated blood samples served as controls. The samples were analyzed and three parameters were drawn from the results at 60 minutes: Maximum clot firmness (stability of the blood clot), clotting time (time to clot formation) and maximum lysis of the blood clot. Assays used were EXTEM, INTEM and FIBTEM. EXTEM measures the activity of the extrinsic pathway with tissue factor activation and INTEM of the intrinsic pathway with contact activation. FIBTEM is used for the assessment of fibrinogen status by complete platelet inhibition.

4.13 Statistical analysis

Results are presented as mean±SD. Student's t-test was used for single comparative analysis and one-way ANOVA with Tukey's or Dunnett's tests for multiple comparisons. A p-value <0.05 was considered statistically significant in all analyses. Kaplan-Meier plots were used for survival analysis.

5 RESULTS

5.1 Survival (I, II and III)

In study I 16 out of the 45 animals in the four-week follow-up died after MI: 6 in the intraperitoneal treatment group, 7 in the intramyocardial treatment group and 3 in the control group. The remaining 29 animals survived for the complete duration of the study. Kaplan-Meier plots did not show statistically significant mortality rates between the groups (p=0.69). In the one-week groups all animals completed the follow-up.

In study II all animals in group 1 died during the first week of follow-up. The deaths were considered to be related to PLD toxicity. In group 2 only one animal died after treatment initiation. In group 3 the mortality rate increased after the third doxorubicin injection. Kaplan-Meier analysis showed no difference in mortality between TB4 treated animals and controls (p=0.27) (Figure 4).

In study III two TB4 treated animals did not complete the follow-up period. One pig died shortly after weaning from cardiopulmonary bypass due to bradycardia and the other one 20 hours after reperfusion due to circulatory failure.

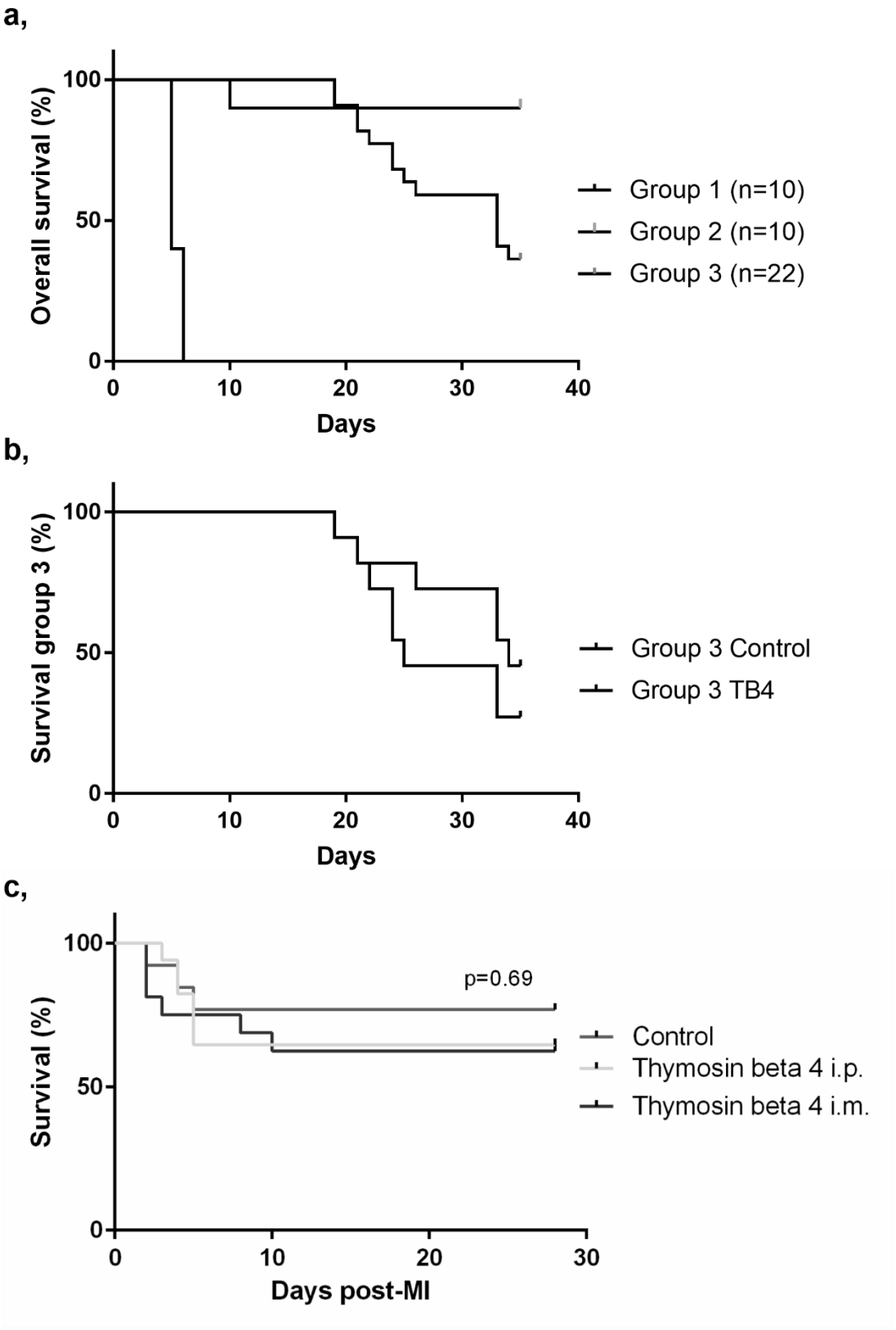

Figure 4. Overall survival (a) and survival in group 3 (b) in study II. Group 1 received a single PLD dose of 20 mg/kg, group 2 a single dose of 5 mg/kg and group 3, 4 weekly doses of 5 mg/kg. In study I the mortality was similar in TB4 treated and control mice 4 weeks after MI (c).

5.2 Cardiac function, remodeling and blood flow (I, II and III)

Echocardiography was performed two days and four weeks after infarction to determine left ventricular remodeling in study I. When comparing ejection fraction (EF), end-systolic volume (ESV), end-diastolic volume (EDV) and MI sizes at the two time-points, no statistically significant differences were observed between the groups. The absolute changes in these parameters over time were however significant in favor of TB4 treated animals. While EF in the control group decreased by 10.1 % during the follow-up period only a 2.0 % decrease in the intraperitoneal treatment group and a 6.7 % increase in the intramyocardial treatment group were observed. Infarct area expansion and increase in ESV and EDV during follow-up was more obvious in control animals than in either treatment group.

In study II the animals in group three underwent serial echocardiography imaging 10, 21 and 35 days after the first PLD injection. Fractional shortening was decreased in control animals at day 10 (26±4 % versus 30±1 %, $p<0.05$) but returned to similar levels as for TB4 treated animals at later time points. No differences in LV internal diameters was observed between the groups and the overall values were consistent between the different time-points.

Cardiac magnetic resonance imaging was performed on pigs in study III after 28 hours of reperfusion. LVEF was slightly higher in the TB4 treated animals but the difference remained statistically insignificant (61.9±10.6 % vs. 56.7±7.6 %, p=0,45). LVEDV (60.1±6.8 ml versus 62.3±3.7 ml, p=0.58) and LVESV (23.3±8.7 ml versus 27.2±6.0 ml, p=0.49) did not differ between the groups. No uptake of gadolinium contrast was observed on late-enhancement scans in any of the animals (Figure 5).

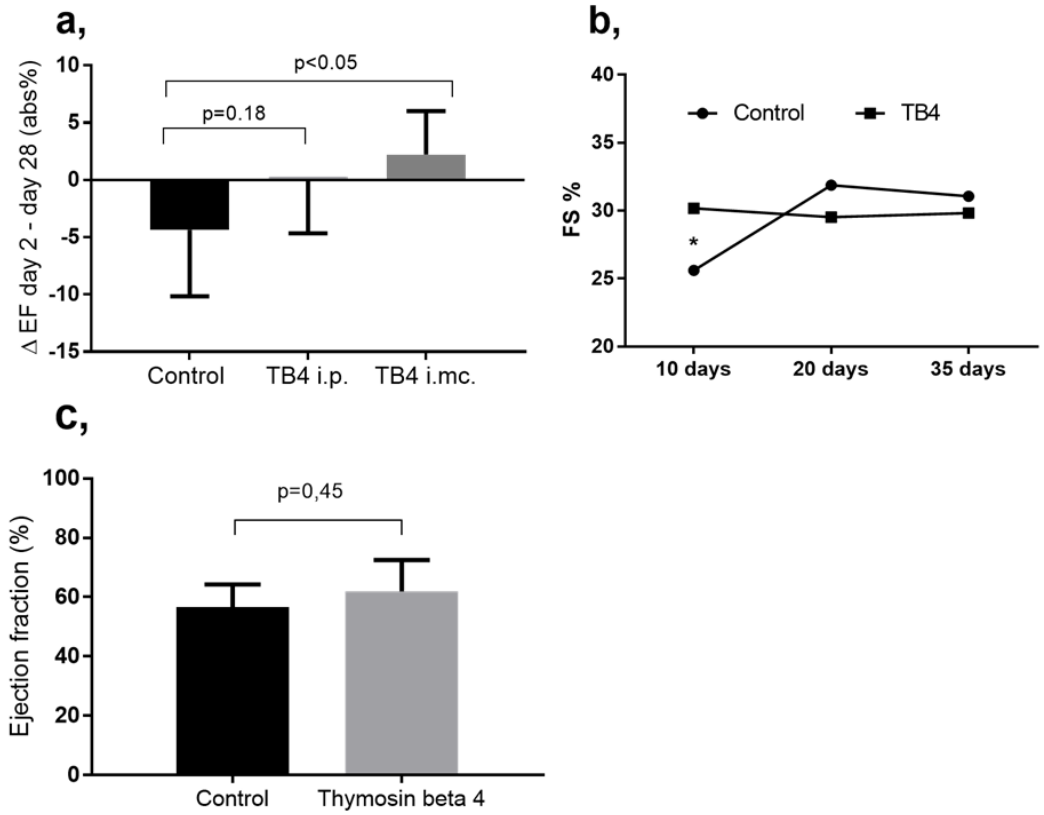

Figure 5. Difference in LVEF at day 2 and day 28 post-MI in controls and TB4 treated mice in study I (a). Fractional shortening (FS) of the LV after PLD given as weekly doses (4 x 5 mg/kg) in controls and TB4 treated mice in study II (b). LVEF 30 hours after I-RI in controls and TB4 treated pigs in study III (c). (*=p<0.05).

Cardiac [15O] H2O positron emission tomography studies were carried out in pigs 25 hours after reperfusion to determine global myocardial blood flow (MBF). Blood flow during rest was equal between TB4 and control group (1.92±0.38 ml/g/min versus 2.01±0,91 ml/g/min, p=0.86). The values were also corrected for individual and group mean blood pressure and pulse rate products but the differences remained insignificant. Coronary flow reserve calculated from flow during adenosine stress and resting flow values were close to 1 in both groups (0.97±0.35 versus 1.15±0.28, p=0.45).

5.3 Myocardial cell proliferation, hypertrophy and fibrosis (I and II)

In study I all samples showed very low numbers of cells positive for the proliferation marker Ki-67 with no differences between the control, intraperitoneal and intramyocardial groups at 4 weeks after MI (0.97±0.12 % versus 1.37±0.38 % (p=0.42) and 1.4±0.58% (p=0.38). Cardiomyocyte sizes, reflecting hypertrophy, were similar at 4 weeks both in the infarct region (27.1±2.2 µm vs. 26.6±3.1 µm (p=0.91) and 24.4±3.4 µm (p=0.09) and in the non-infarcted LV (25.1±2.3 µm vs.

24.6±2 µm (p=0.89) and 25.6±3 µm (p=0.93). In study II tissue samples from the LV were analyzed for myocardial fibrosis 10 and 5 weeks after PLD treatment initiation. At 5 weeks the amount of fibrosis was similar between controls and TB4 treated animals. The total amount of myocardial fibrosis increased by week 10 but the differences between TB4 therapy and controls remained statistically insignificant. Some vacuolization of cardiomyocyte sarcoplasm was observed secondary to PLD cardiotoxicity.

5.4 Myocardial apoptosis, necrosis and inflammation (I and III)

The number of TUNEL-positive apoptotic myocardial cells did not differ at 2 days (7.2±4.5 versus 8.4±1.5 cells/visual field, p=0.75), 5 days (1.2±0.5 vs. 1.1±0.3 cells/visual field, p=0.74) or 7 days (0.7±0.5 vs. 0.6±0.4 cells/visual field, p=0.8) post-MI. There was an increase in CD68 positive macrophages in both groups between day 2 (3.6±1.4 cells/field vs. 1.5±1.1 cells/field, p=0.11), day 5 (11.0±5.1 cells/field vs. 14.6±9.5, p=0.60) and day 7 (19.5±8.4 cells/field vs. 24.8±14.9 cells/field, p=0.62) post-MI without significant differences between the groups.

In study III general histology showed normal tissue architecture with very little inflammatory cell infiltration. The extent of myocardial cell death did not differ between TB4 treated and control animals. TUNEL analysis showed a similar rate of apoptotic cells in both left (0.71±0.2 % versus 0.41±0.3 %, p=0.17) and right (0.63±0.24 % versus 0.94±0.33 %, p=0.2) ventricles. The differences in cTnT plasma concentrations at six (437±291 ng/l versus 1100±1053 ng/l, p=0.27) and twenty-four hours (305±75 ng/l versus 349±114 ng/l, p=0.55) post-reperfusion did not reach statistical significance (Figure 6).

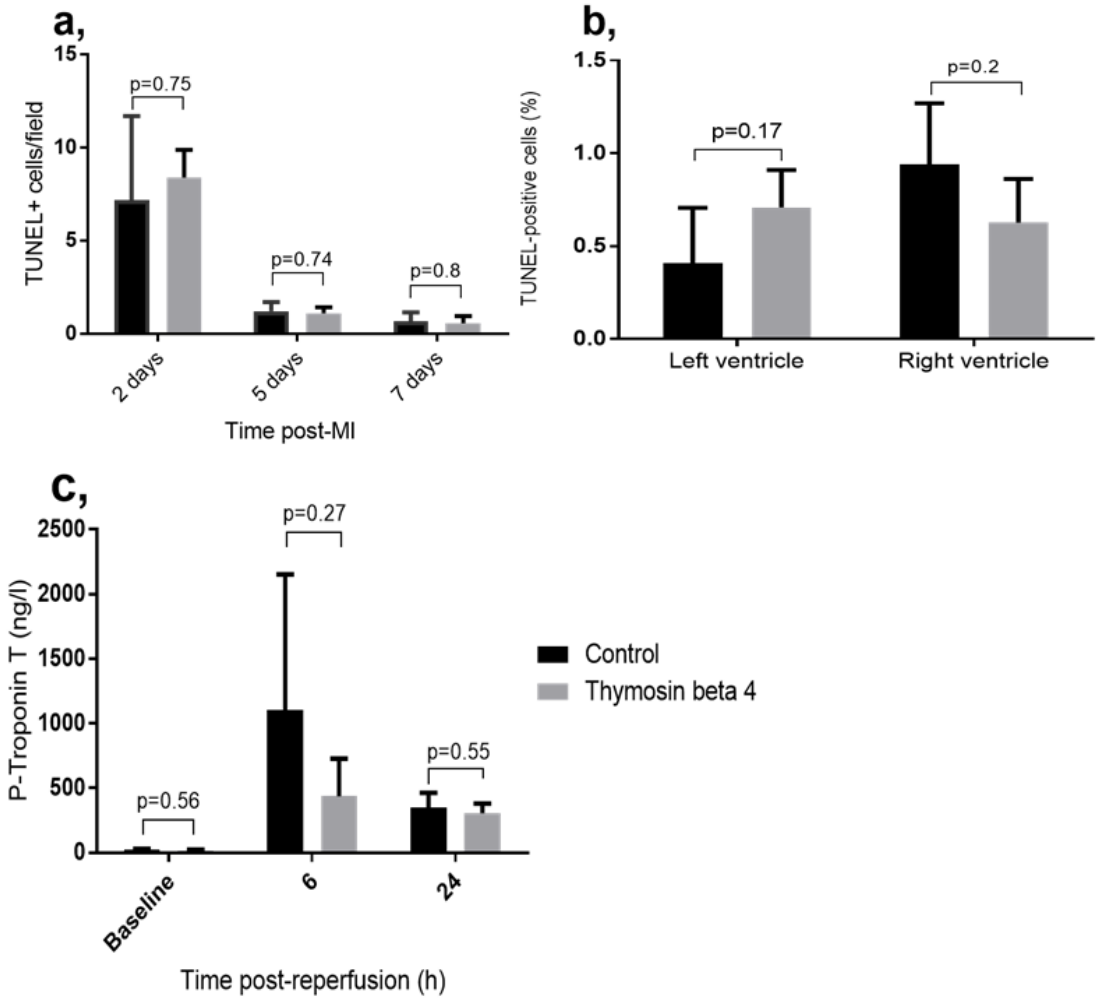

Figure 6. TUNEL positive cells 2, 5 and 7 days after MI in controls and TB4 treated mice in study I (a). TUNEL positive cells (b) 30 hours after I-RI in pigs and cTnT release at 6 and 24 hours in study III (c).

5.5 Epicardial gene expression and chitinase 3-like-1 (I)

To get further insight on the protective mechanisms of TB4, we carried out whole genome gene expression analysis on cardiac tissue samples after MI. At 2 days post-MI there was significant upregulation of four genes in TB4 treated animals compared to controls. Two of the genes, start domain containing 10 (Stard10) and chitinase 3-like-1 (ch3l1), have previously been characterized as epicardial signature genes [10]. The other, CD209f and coiled-coil domain containing 80 (ccdc80) are in turn related to dendritic cell-mediated endocytosis and modulation of glucose and energy homeostatis, respectively. We validated the upregulation of Ch3l1 with qRT-PCR, which showed a 4-fold increase in mRNA expression at 2 days ($p=0.07$) and a 2-fold increase at 7 days ($p<0.05$) post-MI compared to controls. The amount of Ch3l1 positive cells was slightly higher in TB4 treated animals at

2 (17.8±3.9 cells/field vs. 22.0±5.6 cells/field, p=0.34), 5 (19.1±9.5 cells/field vs. 33.3±14.6, p=0.23) and 7 days (21.9±11.5 cells/field vs. 26.7±10.1 cells/field, p=0.62) after MI but the differences remained statistically insignificant (Figure 7).

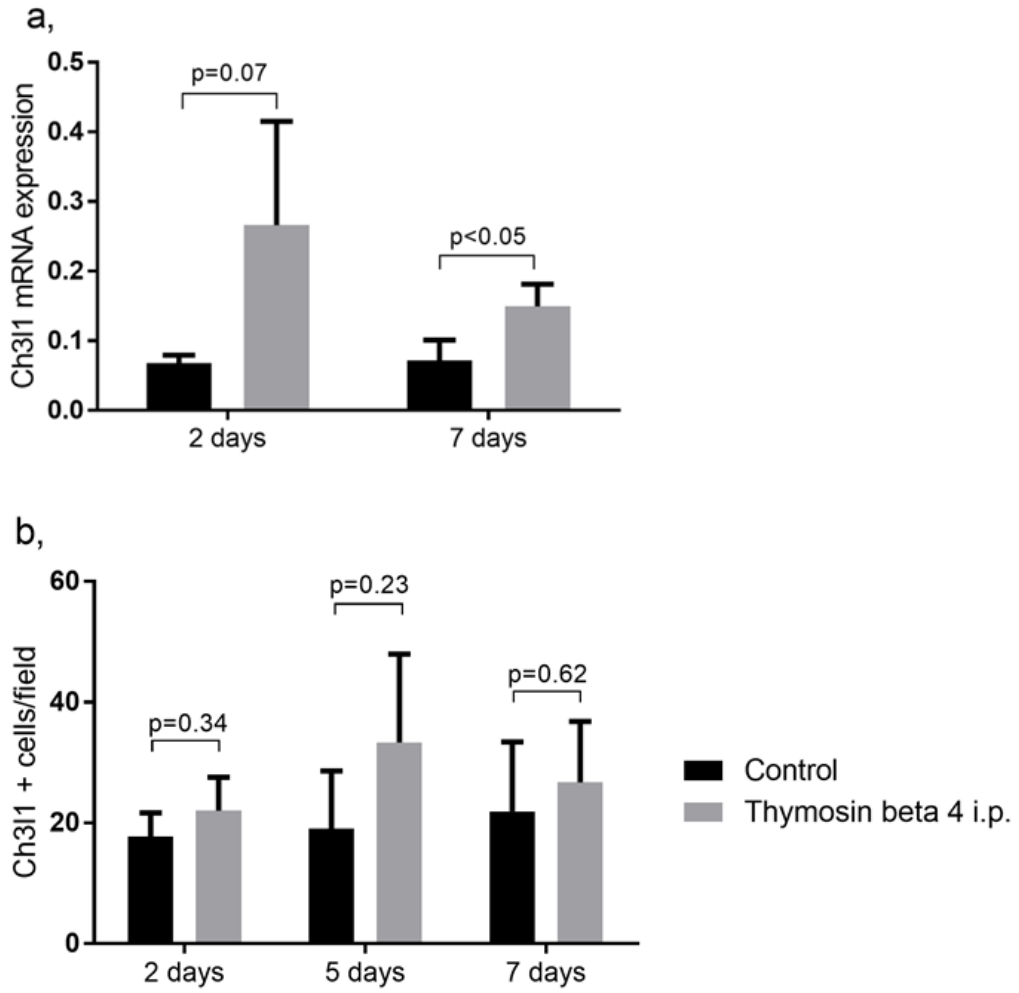

Figure 7. Ch3l1 mRNA expression (a) and ch3l1 positive cells on immunohistochemistry (b) in control and TB4 treated mice after MI (study I).

5.6 ATP/ADP levels and purinergic ecto-enzyme activity (I)

To investigate the involvement of purinergic signaling in TB4 cardioprotection we screened plasma samples 2, 5 and 7 days after MI. Plasma ATP and ADP levels were similar between the groups at all time points and the activity of soluble ATP and ADP hydrolyzing enzymes remained unaltered at all three time points. The activity of CD73, which converts AMP to adenosine increased significantly in TB4 treated animals over time and was significantly higher at 7 days post-MI compared to controls (483±47 vs. 643±76 µmol/h/ml, p<0.05). The enzymes adenylate ki-

nase (AK) and nucleoside-diphosphate kinase (NDPK), which catalyze the production of ADP and ATP, showed similar and consistent levels of activity in all samples.

5.7 TB4 serum concentrations (III)

In the pig study EIA showed increased serum concentrations of TB4 after each dosage. The peak concentration occurred 15 minutes after the first dose (0.96 µM) and a second peak was observed at the start of reperfusion (1.5 µM) approximately 3 hours after the first dose. An increase, although smaller than in the treated animals, of endogenous TB4serum concentration could be seen in control animals immediately after reperfusion (0.49 µM). One hour after the second dose the serum concentration of TB4 was again elevated (1.25 µM), while it remained low in control samples.

5.8 Thymosin beta 4 in vasomotor control and coagulation (III)

Artery wire myography showed a dose-dependent increase in *in vitro* vasoconstriction of pig mesenteric vessels when the samples were treated with TB4 solution under the influence of L-NNA. At concentrations of 0.1mM – 1 mM the contractile response was 15-30 % of that of maximum potassium chloride stimulation. TB4 did not present marked vasoconstriction effects on coronary arteries and only modest NO-dependent vasodilation. The addition of TB4 to whole blood did not have an effect on blood clotting time or maximal blood clot fibrinolysis at concentrations used here. The clot firmness was however significantly reduced in the FIBTEM analysis, which measures clot firmness in the absence of functioning platelets, when comparing 1 mM to 0.1 mM and 0 mM concentrations (27.7±4.2 mm versus 37.3±5.9 mm versus 40.6±9.0 mm, $p<0.05$) (Figure 8).

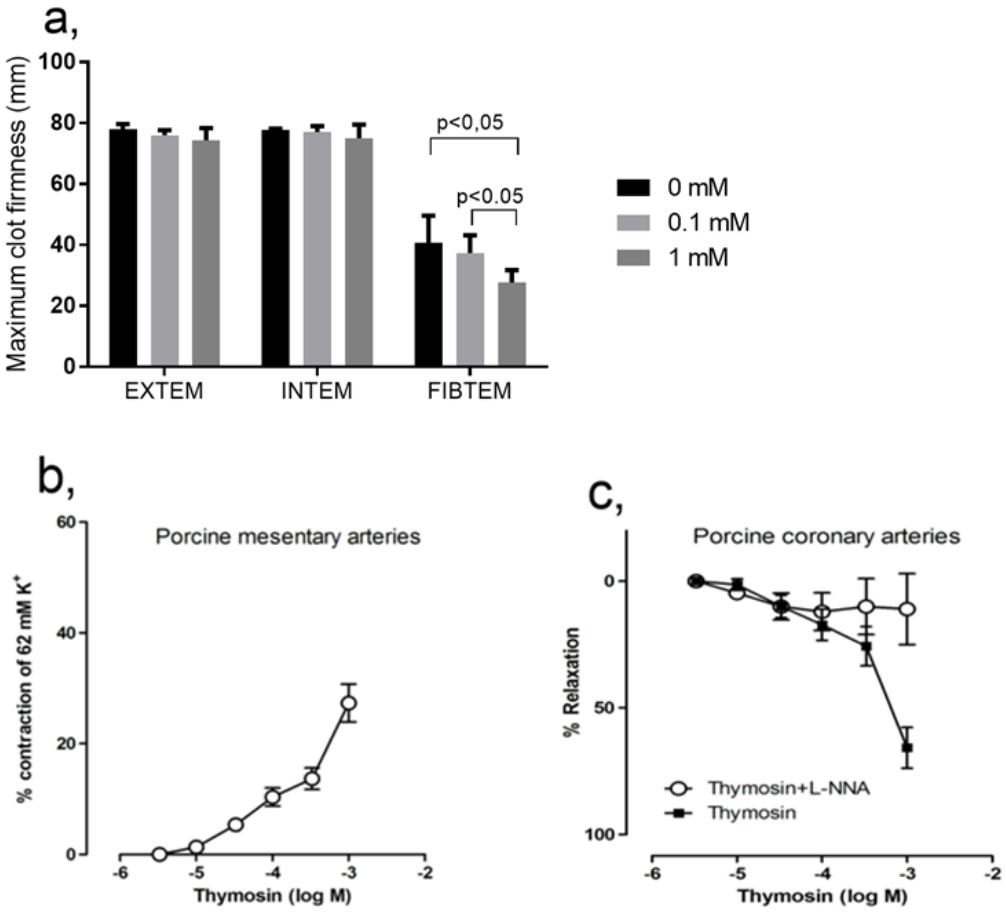

Figure 8. TB4 decreases blood clot firmness after platelet inhibition (a) and induces mesentery artery vasoconstriction (b) and mild coronary vasodilation (c) at high concentrations in pigs (study III).

6 DISCUSSION

6.1 Appropriateness of the animal models used

We aimed to first reproduce the efficacy of TB4 treatment seen in earlier studies. For further translational evaluation, we sought to demonstrate efficacy in other clinically relevant animal models as well. This highlights the diversity of the peptides' biology and possible applications although it makes comparison between studies more challenging.

The mouse experiments were all performed on male FVB/n mice. Previous studies with TB4 have mostly used the mouse strain C57Bl/6. The cardiac phenotypes of these mouse strains are similar. FVB/n mice have been shown to possess innate cardioprotection from short periods of ischemia and reperfusion. With longer ischemic times the amount of damaged myocardium is however similar to C57Bl/6 mice. In the setting of total coronary occlusion and MI there could be strain related differences in infarct size and impairment of cardiac function, which could partly explain the modest protective effect of TB4 in our studies compared to previous reports [Li Q et al 2006, Guo Y et al 2012]. It has also been shown that the incidence of LV rupture is substantially less frequent in FVB/n mice after MI [Gao X et al 2005]. Study I of this thesis showed a 36 % mortality after MI and the majority of the animals had ruptured LV walls. If this is related to TB4 treatment remains unclear. Since there was a protective effect of TB4 in study I, we considered this mouse strain to be suitable for the cardiomyopathy model in study II. Based on the known cardiotoxicity of doxorubicin we hypothesized that TB4 would be effective in attenuating these effects. The decision to use the PLD formulation of the drug was based on expected survival benefit. It, however turned out to be less safe and less cardiotoxic and ultimately inappropriate for inducing cardiomyopathy.

In a recent study TB4 was cardioprotective after unprotected local I-RI in a pig model. Therefore, we considered the CPB assisted pig model for global I-RI to be suitable for further evaluation of the peptide. This model is commonly used but has some limitations addressed in earlier chapters of this thesis. In this study, we assessed cardioprotection by several biochemical and functional analyses and concluded that TB4 did not offer any notable therapeutic effect. This was interpreted as a failure of therapy and not failure of the experimental model itself.

6.2 Rationale for the use of thymosin beta 4 as a cardioprotective agent

Based on the cardioprotective findings in earlier trials there is strong preclinical evidence that TB4 treatment is beneficial after myocardial infarction and possibly

in attenuating ischemia-reperfusion injury. The peptide has been shown to be well tolerated after intravenous use in healthy human volunteers [Ruff et al 2010]. In our studies, we did not observe any adverse effects of the peptide. There was no influence of TB4 treatment on mortality in any of our studies although TB4 has previously been shown to reduce death after MI in rodents [Peng et al 2014]. The efficacy of the peptide still needs to demonstrate favorable impact on hard end-points, such as mortality also in large animals or human trials.

6.3 Dosing of thymosin beta 4 in cardioprotection

In our studies, we used the commonly accepted single TB4 dosing of 6 mg/kg in all experiments with systemic administration. Local injections were given after MI into the peri-infarct area of the LV at doses of 400 ng. There was slightly better cardiac performance after local myocardial delivery compared to intraperitoneal administration although the cumulative dose was much smaller and given less frequently. After PLD-induced cardiomyopathy we failed to show any significant effect of TB4 treatment. In pigs, intravenous delivery before and after ischemia-reperfusion showed no therapeutic effect measured with several parameters for cardioprotection. This could indicate that systemic doses of TB4 should be higher in order, to achieve cardioprotection. It is also possible that the animal models used in studies II and III caused too little myocardial damage for any therapeutic effects to be seen. We did however demonstrate increased serum concentrations of TB4 in pigs after delivery. If this also translates into higher heart tissue concentrations is unclear. The optimal cardioprotective serum concentrations are uncertain and still needs to be addressed when planning systemic delivery in future studies [Morris et al 2014, Bollini et al 2015].

6.4 Functional outcome after myocardial injury and thymosin beta 4 treatment

TB4 was potentially cardioprotective after MI. The effect on LV dilatation and reduction in EF was however smaller than in previously reported studies which could be related to dosing strategies, mouse strain differences or more specific and sensitive imaging [Bock-Marquette et al 2004, Barnabei et al 2010, Sopko et al 2011, Smart et al 2011, Stark et al 2012, Bao et al 2013]. After PLD treatment left ventricular fractional shortening was slightly higher in TB4 treated animals at 10 days after the first PLD dose. The absolute change was small and at later time-points the difference disappeared. Although limited by the lack of substantial PLD cardiotoxicity, it is unlikely that this finding has any practical significance. In pigs LVEF was reduced after global I-RI with a slight, although statistically insignificant better preservation of function in TB4 treated animals. There was also a trend for larger diastolic and systolic dimensions of the LV in control animals. Previous data is encouraging but to date only a few studies have been performed using large animal models in cardioprotective studies with TB4. In these studies, TB4 has been

injected directly into the heart or over-expressed by adenoviral vector transfer of TB4 [Hinkel et al 2008, Postrach et al 2014].

6.5 Myocardial blood flow after global ischemia-reperfusion injury

A no-reflow phenomenon is associated with cardiac ischemia-reperfusion injury and we wanted to determine global myocardial perfusion and possible effects by TB4 treatment in the subacute phase of I-RI. Coronary flow reserve was low in both study groups with no difference in global MBF between TB4 treated pigs and controls. The observed correlation between cTnT levels in plasma and MBF during rest could indicate an association between the extent of cell injury and hyperemia. In preclinical models using unprotected local myocardial I-RI injury, blood flow is decreased early after reperfusion and remains low during the first 24 hours. In studies with protected global I-RI, MBF has been shown to increase immediately after ischemia, then decrease within the first hours of reperfusion and later rise to normal or even higher levels. In some situations, this hyperdynamic blood flow is still observed several days after surgery, the clinical significance of this is however unclear [McFalls et al 1995, Awurabi et al 2009]. Earlier measurements of MBF might have showed signs of malperfusion and could have highlighted some differences in favor of TB4.

6.6 The influence of thymosin beta 4 on cardiomyocyte cell death

After MI, there was no differences in cardiomyocyte apoptosis during the first week between controls and TB4 treated mice. The incidence of apoptotic cells decreased between days two and seven. Peak apoptosis rate is reached during the first 24 hours after MI and analyzing cell death earlier might have been useful [Saraste et al 2007]. In pigs, there was substantial cardiomyocyte injury following I-RI as demonstrated by the increase in post-operative cTnT levels and the rate of apoptosis. TB4 did however not influence the rate of myocardial cell death. There are no previous studies using protected global myocardial I-RI models as we did in this study. After unprotected local I-RI, TB4 has been shown to reduce myocardial cell death but the mechanisms of apoptosis and necrosis in these two situations are different and could at least partly explain the lack of cell survival benefit after TB4 treatment [Hinkel et al 2008].

6.7 Cardiac inflammation and fibrosis

The amount of CD68 positive cells increased gradually from day 2 to day 7 after MI without differences between groups. In a previous study, it was reported that the peak influx of macrophages occurs four days after MI in TB4-sulfoxide treated mice and then decreased rapidly within the first week [Evans et al 2013]. The authors used a marker for M1 polarized macrophages as we also did in our study.

Therefore, the decrease in positive cells could also have been related to a switch in phenotype rather than a true decrease in macrophage number. We did not further investigate the phenotype of the cells in this study. This could have been valuable in order, to identify pro-inflammatory (M1) and cardioprotective or reparative (M2) macrophages.

After PLD treatment the amount of myocardial fibrosis was slightly higher at 10 weeks although the animals only received half the cumulative dose (10 mg/kg versus 20 mg/kg) as compared to the group studied at 5 weeks. This could indicate that myocardial fibrosis is a time-dependent process rather than just related to dose after PLD treatment. TB4 did not significantly impact the amount of fibrosis, although it has previously been associated with a reduction in myocardial fibrosis after ischemic heart injury [Goldstein et al 2012, Yan et al 2013].

6.8 Gene-expression after myocardial infarction

We observed upregulation of four genes after MI in TB4 treated mice. CD209f, a marker for antigen presenting dendritic cells has a crucial role in regulating the inflammatory response after MI and was upregulated in TB4 treated animals. After MI in humans the amount of myocardial CD209-positive cells correlates negatively with ventricular rupture and is associated with increased reparative fibrosis [Nagai et al 2014]. Stard10 is involved in intracellular lipid transfer and has also been shown to be a signature epicardial gene that is downregulated after MI in mice [Bochmann et al 2010]. Several genes considered epicardial markers were in our study up-regulated in TB4 treated animals. This finding could support previous findings on TB4 induced epicardial progenitor cell activation.

Ch3l1 or YKL-40 is a chitin binding protein secreted by macrophages, neutrophils and vascular smooth muscle cells and has recently gathered attention as a marker for inflammation and fibrosis [Kastrup 2012]. The proteins' upregulation on microarray analysis was confirmed by RT-PCR, where mRNA levels for Ch3l1 were higher in treated animals at days 2 and 7 post-MI compared to controls. Immunohistochemistry for Ch3l1 showed slightly more positive cells in the infarct area of TB4 treated animals at all three time points. In previous trials increased plasma levels of Ch3l1 has been found in patients during the first week after myocardial infarction and in some reports this elevation was still observed one month after the event. One study showed a negative correlation between maximal Ch3l1 levels and left ventricular EF recovery after MI [Hedegard et al 2010]. In our study, peak mRNA expression for Ch3l1 was observed in TB4 treated animals 2 days after MI and remained higher at day 7 post-MI. The increase was also seen on histology. The different profile in Ch3l1 expression in the two groups could be related to TB4-mediated modification of the inflammatory response after MI. Comparing our finding with previous clinical findings is difficult as the source of ch3l1 is likely

different. The survival signaling protein Akt is a common target for both TB4 and ch3l1. Both induce Akt phosphorylation in a PI3K-dependent manner but TB4 also regulates its phosphorylation through ILK-PINCH-1 activation. Wortmannin is a potent inhibitor of PI3K and in studies with TB4 it has been shown to abolish the infarct size reducing effects of TB4 with only minimal decrease in myocardial function. This suggests that the different phosphorylation mechanisms lead to different downstream signaling and effector activation or that TB4 offers cardioprotection through Akt-independent pathways. There are several similarities between the functions of TB4 and ch3l1. Both proteins are highly conserved but without significant similarities in their amino acid sequences. The combined effects of TB4 and Ch3l1 could in theory enhance Akt-mediated signaling and further improve cardiomyocyte survival. The role of ch3l1 upregulation still needs to be assessed and confirmed in future studies.

6.9 Purinergic signaling

Recently, it was reported that TB4 increases extracellular ATP synthesis and possibly induces purine receptor-mediated endothelial cell migration [Freeman et al 2011]. Therefore, we screened plasma samples for ATP and ADP levels and for activities of soluble purine-converting enzymes. ATP is not able to diffuse across the cell membrane but is released after cellular damage or through several different mechanisms. In the heart, ATP acts mainly on P2-receptors causing vasoconstriction and increased inotropy, but also induces arrhythmia. ATP is a powerful chemoattractant for leukocytes and signaling through P2x7-receptors induces inflammatory and apoptotic pathways. ATP is metabolized to ADP and further to AMP by different hydrolyzing enzymes, collectively called ATPases and ADPases. AMP in turn is hydrolyzed to adenosine by CD73. ADP, is able to reduce ischemic damage in the heart by acting on P2y-receptors but is also a key transmitter in thrombosis formation. Adenosine in turn has shown several cardioprotective properties. In this study plasma ATP and ADP levels were similar between the groups with a trend for higher overall levels at day 7 post MI. ATPase and ADPase activities were consistent during the first week. In TB4 treated animals the activity of CD73 continued to increase from day 2 to day 7 after infarction and at day 7 the activity was significantly higher than in controls. After myocardial damage, mainly inflammatory cell-derived CD73 is responsible for the local increase in Adenosine. This CD73-derived adenosine in turn reduces inflammation and can also enhance the activation of anti-inflammatory M2 macrophages, possibly suppressing the inflammatory reaction further [Bönner et al 2013]. Based on these findings, TB4's influence on purinergic signaling may not be limited to ATP synthase but might also involve other enzymes.

6.10 Vasoreactivity and blood coagulation

As the functions of TB4 are thought to be related to tissue homeostasis and repair after injury, we wanted to test the direct vasomotor effect of peptide in an in vitro setting. When using physiological concentrations of TB4 we did not observe any influence on vasoconstriction but when the dose was adjusted to concentrations in the 0.1-1.0mM range we saw a clear increase in vasomotor tone in mesentery arteries. Vascular smooth muscle tone is usually regulated by intracellular Ca2+ and mediated by several cell surface receptors. A previous report showed an increase in intracellular Ca2+ after treatment with TB4 but this was not confirmed in a more recent study [Cerniewski et al 2012]. Purinergic signaling through P2 receptors is associated with vasomotor responses and is regulated either in the endothelium or by sympathetic nerve stimulation in the vessel wall [Burnstock 2008]. No previous data exists on TB4 mediated vasoconstriction and we can only speculate whether this has any clinical significance as plasma levels are normally much lower compared to doses tested here. Platelets however contain up to 0.5mM of TB4 and at sites of blood clot formation local concentrations could increase sufficiently to cause a vasoconstrictor response mediated by known or still undiscovered targets of TB4.

It is documented that TB4 covalently binds to fibrin and fibrinogen by factor XIIIa and that his interaction inhibits platelet aggregation and adhesion at concentrations higher than 0.5 µM [Kaur et al 2012]. Based on the findings in our vasomotor experiment, we investigated whether even higher concentrations of TB4 had an influence on blood coagulation as assessed by thromboelastometry. We observed a decrease in maximal blood clot firmness with increasing doses from 0.1 – 1.0mM when platelets were inhibited by a potent anti-platelet agent. This method is used to assess fibrinogen status in whole blood and could indicate that the fibrinogen-TB4 interaction has a negative effect on blood clot stability and might be relevant in surgical situations where the patient is also receiving anti-platelet medication.

6.11 Translation into clinical medicine

TB4 treatment leads to various degrees of improvement in cardiac function and reduction in cell death after MI in rodents. The literature also suggests that it increases angiogenesis and influences the inflammatory milieu in injured myocardium leading to blunting of inflammation and increased regeneration either through reparative fibrosis or true tissue regeneration. In this thesis, we focused on TB4-mediated cardioprotection and reproduced some of the positive effects seen on cardiac function after MI but observed no reduction in inflammation or cell death. In the pig study, we were unable to show any influence of TB4 on cardiac cell death, blood flow or function. One hurdle in the translation of TB4 therapies is poor knowledge of the peptides' pharmacokinetics. Optimal administration routes and dosing still need to be determined. The opinion of the author is that

further studies with clinically relevant large animal models demonstrating efficacy on the parameters mentioned above, should be considered before proceeding into clinical trials.

6.12 Limitations

In these studies, we did not measure activities of known TB4-related pathways such as Akt phosphorylation. We did however demonstrate efficacy and displayed a therapeutic window for TB4 therapy after MI. Despite small sample sizes we demonstrated significant changes in some of the parameters measured. After PLD treatment there was unexpected high mortality which caused variability in the dosing protocols impacting comparison of the study groups. Based on the echocardiographic findings PLD administration was not severely cardiotoxic and therefore any possible therapeutic effect of TB4 is likely to be unrecognizable. In the I-RI experiment pigs were kept on mechanical ventilation for quite a long time and the animals received several inotropic agents. The use of these drugs was often necessary, in order to successfully wean the animals from CPB as it also is in clinical situations. Keeping the animals sedated for the whole follow-up period was considered appropriate as recovery from anesthesia and re-intubation for imaging procedures would have caused additional stress to the animals increasing the possibility for complications such as hypoxemia and arrhythmias. The number of animals in the pig study was low and the effect size of the observed differences was small but increasing animal number would probably not have emphasized the results.

7 CONCLUSIONS

1. TB4 treatment with intramyocardial delivery slightly reduced remodeling and improved cardiac function after MI in mice.

2. Ch3l1 was upregulated and CD73 activity increased after MI in TB4 treated mice.

3. The use of PLD was not appropriate for inducing experimental cardiomyopathy as no substantial impairment of cardiac function was observed and the treatment was associated with high overall mortality.

4. PLD treatment caused some myocardial fibrosis but its impact on cardiac function remains unclear. Thymosin beta 4 did not significantly influence the amount of fibrosis after PLD treatment.

5. Systemic TB4 treatment was not beneficial after global myocardial ischemia-reperfusion injury in pigs.

6. High doses of TB4 is related to blood coagulation and influences blood clot stability.

7. TB4 causes vasoconstriction in mesentery arteries and mild vasodilation in coronary arteries at high concentrations.

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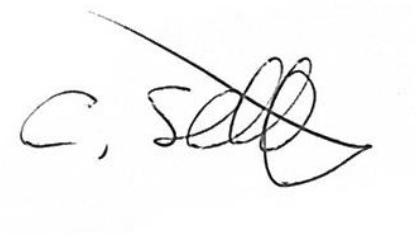
Sincerely,



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Helsinki 5.9.2017

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