Cardioprotective treatment strategies

vanderPals, Jesper

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JESPER VAN DER PALS, MD

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Faculty Opponent
Professor Dan Atar, Department of Cardiology
Oslo University Hospital, Ullevål, Norway

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Author(s) Jesper van der Pals

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Abstract

In myocardial ischemia-reperfusion (I/R) injury, complement activation, tissue plasminogen activator (t-PA) and extracellular adenosine triphosphate (ATP) release contribute to myocardial injury. ATP is degraded into adenosine by the enzyme apyrase, and adenosine possesses cardioprotective properties. ADC-1004 is an antagonist of the receptor to the activated complement factor C5a. Hypothermia has been shown to suppress the development of I/R injury. In this thesis, the cardioprotective effects of ADC-1004 (paper I), apyrase (paper II) and hypothermia (paper III) were investigated. The effects of hypothermia on coronary t-PA release (paper IV), and on systemic t-PA release in cardiogenic shock (paper V) were also studied. An experimental porcine ischemia/reperfusion model was used. Infarct size (IS), microvascular obstruction and area at risk (AAR) were measured with ex-vivo MRI and SPECT.

ADC-1004 treatment (paper I) was found to reduce infarct size (ADC-1004: 58.3±3.4 vs control: 74.1±2.9 %AAR, p=0.007) but not microvascular obstruction (ADC-1004: 2.2±1.2 vs control: 5.3±2.5 %AAR, p=NS). Treatment with apyrase (paper II) did not reduce infarct size (apyrase: 75.7±4.2 vs saline: 69.4±5.0 %AAR, p=NS) nor microvascular obstruction (apyrase: 10.7±4.8 vs saline: 11.4±4.8 %IS, p=NS). Hypothermia (paper III) reduced both infarct size (hypothermia: 60.8±4.9 vs normothermia: 73.8±4.0 %AAR, p<0.05) and microvascular obstruction (hypothermia: 0.5±0.5 vs normothermia: 21.5±5.2 %IS, p<0.001). Hypothermia also inhibited an increase in coronary net t-PA release during reperfusion (paper IV; hypothermia: 0.79±0.45 ng/ml vs normothermia: 9.44±4.34 ng/ml, p<0.05); and an increase in systemic net t-PA release in cardiogenic shock (paper V; hypothermia: 0.60 ± 0.12 ng/ml vs normothermia: 2.16 ± 1.09 ng/ml, p<0.05).

In conclusion, complement inhibition by ADC-1004 and therapeutic hypothermia reduces myocardial ischemia-reperfusion injury, and represents clinically applicable treatment strategies. Mechanistically, therapeutic hypothermia acts to reduce t-PA release in myocardial ischemia and cardiogenic shock. Treatment with apyrase does not protect the heart from ischemia/reperfusion injury.

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JESPER VAN DER PALS, MD

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Cover: The C5a receptor antagonist ADC-1004. Illustration made by Björn Walse, SARomics AB, Sweden. Published with permission from copyright holder, Alligator Bioscience AB, Sweden.

To my family

Contents

ABSTRACT

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ADC-1004 treatment (**paper I**) was found to reduce infarct size (ADC-1004: 58.3±3.4 vs control: 74.1±2.9 %AAR, p=0.007) but not microvascular obstruction (ADC-1004: 2.2±1.2 vs control: 5.3±2.5 %AAR, p=NS). Treatment with apyrase (**paper II**) did not reduce infarct size (apyrase: 75.7±4.2 vs saline: 69.4±5.0 %AAR, p=NS) nor microvascular obstruction (apyrase: 10.7±4.8 vs saline: 11.4±4.8 %IS, p=NS). Hypothermia (**paper III**) reduced both infarct size (hypothermia: 60.8±4.9 vs normothermia: 73.8±4.0 %AAR, p<0.05) and microvascular obstruction (hypothermia: 0.5±0.5 vs normothermia: 21.5±5.2 %IS, p<0.001). Hypothermia also inhibited an increase in coronary net t-PA release during reperfusion (**paper IV**; hypothermia: 0.79±0.45 ng/ml vs normothermia: 9.44±4.34 ng/ml, p<0.05); and an increase in systemic net t-PA release in cardiogenic shock (**paper V**; hypothermia: 0.60 ± 0.12 ng/ml vs normothermia: 2.16 ± 1.09 ng/ml, p<0.05).

In conclusion, complement inhibition by ADC-1004 and therapeutic hypothermia reduces myocardial ischemia-reperfusion injury, and represents clinically applicable treatment strategies. Mechanistically, therapeutic hypothermia acts to reduce t-PA release in myocardial ischemia and cardiogenic shock. Treatment with apyrase does not protect the heart from ischemia/reperfusion injury.

LIST OF PUBLICATIONS

The thesis is based on the following papers, referred to in the text by their roman numerals.

I. J van der Pals, S Koul, P Andersson, M Götberg, J Ubachs, M Kanski, H Arheden, G Olivecrona, B Larsson and D Erlinge. Treatment With the C5a Receptor Antagonist ADC-1004 Reduces Myocardial Infarction in a Porcine Ischemia-Reperfusion Model. BMC Cardiovasc Disord 2010 Sep 27;10:45.

II. J van der Pals, S Koul, MI Gotberg, GK Olivecrona, M Ugander, M Kanski, A Otto, M Gotberg, H Arheden and D Erlinge. Apyrase treatment of myocardial infarction according to a clinically applicable protocol fails to reduce myocardial injury in a porcine model. BMC Cardiovasc Disord 2010 Jan 4;10:1.

III. M Götberg, J van der Pals, M I Götberg, G Olivecrona, M Kanski, S Koul, A Otto, H Engblom, M Ugander, H Arheden and D Erlinge. Timing of hypothermia in relation to myocardial reperfusion. Submitted.

IV. J van der Pals, M Gotberg, GK Olivecrona, H Brogren, S Jern and D Erlinge. Mild Hypothermia Markedly Reduces Ischemia Related Coronary t-PA Release. J Thromb Thrombolysis 2010 Apr;29(3):289-95.

V. J van der Pals, MI Gotberg, M Gotberg, L Mattsson Hultén, M Magnusson, S Jern and D Erlinge. Hypothermia in cardiogenic shock reduces systemic t-PA release. J Thromb Thrombolysis. In press.

LIST OF ABBREVIATIONS

AAR = area at risk

AMP = adenosine monophosphate

ATP = adenosine tri-phosphate

C5a = activated complement factor C5a

CHIPS = chemotaxis inhibitory protein of Staphylococus aureus

IL-6 = interleukin 6

IL-10 = interleukin 10

iNOS = inducible nitric oxide synthase

LAD = left anterior descending artery

LVM = left ventricular myocardium

MO = microvascular obstruction

MPTP = mitochondrial permeability transition pore

MRI = magnetic resonance imaging

NO = nitric oxide

I/R = ischemia / reperfusion

IS = infarct size

SPECT = single photon emission computed tomography

STEMI = ST-elevation myocardial infarction

TGF-β = transforming growth factor beta

TNF-α = tumour necrosis factor alpha

t-PA = tissue plasminogen activator

INTRODUCTION

The unifying theme in this thesis is cardioprotection. Cardioprotection denotes the protection of the myocardium against injury caused by ischemia and subsequent reperfusion. Tissue damage after ischemia-reperfusion (I/R) is called I/R-injury, figure 1.

Ischemia leads to cessation of aerobic metabolism, resulting in depletion of high-energy phosphate compounds and accumulation of potentially noxious metabolites such as lactate. Sodium, calcium and hydrogen ions also accumulate in the cardiomyocytes, culminating in tissue acidosis. Ultrastructural changes, such as organelle swelling and cytoskeletal damage, follows. With prolonged ischemia, the integrity of the cellular membrane is disrupted, leading to necrosis and leakage of cellular macromolecules into the cardiac intestitium, lymphatics and vasculature.[1, 2]

The development of ischemic injury is brought to a halt by reperfusion, which is a prerequisite for salvage of ischemic myocardium. Paradoxically, reperfusion is also thought to induce tissue injury.

The concept of reperfusion injury was proposed by Jennings et al over 50 years ago, when they noted that reperfusion of canine hearts subject to coronary ligation appeared to accelerate necrosis.[3] Despite an improved understanding of reperfusion injury, effective therapies have proven elusive and the phenomenon is under on-going debate. However, adjunctive therapies to limit reperfusion injury remain an active area of investigation.

Prolonged I/R injury to the heart results in infarcted tissue, figure 2. Infarct size (IS) is a major determinant of mortality and morbidity.[4-6] I/R injury may also damage the myocardial capillaries to the degree that even with restoration of epicardial blood flow, the capillary bed does not reperfuse. This is called microvascular obstruction (MO). The development of MO is a multifactorial process attributable to endothelial damage, thrombus formation, neutrophil aggregation, myocyte swelling, capillary spasm and debris from dying cells.[7, 8] MO has been found to be a strong independent predictive marker of post-infarction complications even after adjustment for infarct size.[9, 10]

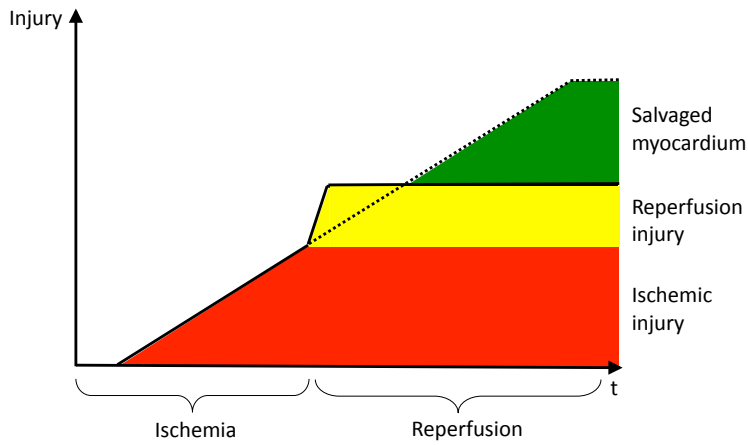

Figure 1: Development of ischemia/reperfusion injury
Ischemia causes tissue necrosis. Reperfusion brings ischemic cell death to a halt, and is a prerequisite for salvage of the ischemic myocardium. However, reperfusion also causes additional damage to the previously ischemic myocardium, i.e. reperfusion injury. The component of the total injury that is attributable to reperfusion injury could be available for adjunctive therapy.

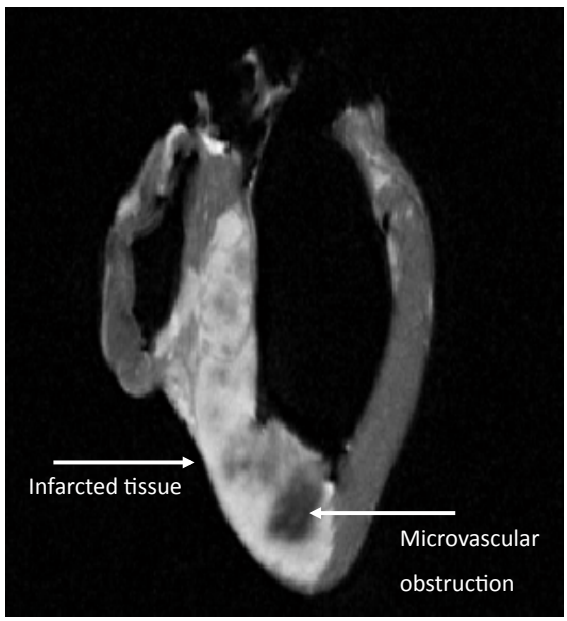

Figure 2: Components of myocardial injury
Delayed contrast enhanced long axis MR image showing infarcted myocardium (white) and microvascular obstruction (hypointense region in the core of the infarction). Prognosis and complications depend on the extent of both infarct size and microvascular obstruction.

Cardiogenic shock may result from myocardial I/R injury, and is a state of inadequate systemic tissue perfusion, despite adequate left ventricular filling pressure. It is caused by extensive myocardial damage and appears to be aggravated by a systemic inflammatory response.[11-14] The result is hypotension with metabolic acidosis and often a fatal outcome.

Mechanisms of reperfusion injury

The molecular and cellular mechanisms that underlie reperfusion injury are complex, intertwined and have not been fully elucidated. Manifestations are considered to include myocyte death, cell damage leading to microvascular dysfunction, reperfusion arrythmias and myocardial stunning.[15]

Factors that contribute to reperfusion injury include damage to organelle membranes including mitochiondria, myocyte hyperconctracture, free radical formation, aggregation of leukocytes and inflammatory mediators, platelet activation, complement and pro-apoptotic signalling cascade activation, endothelial damage, vasoconstriction and distal microembolization, figure 3.[1, 15]

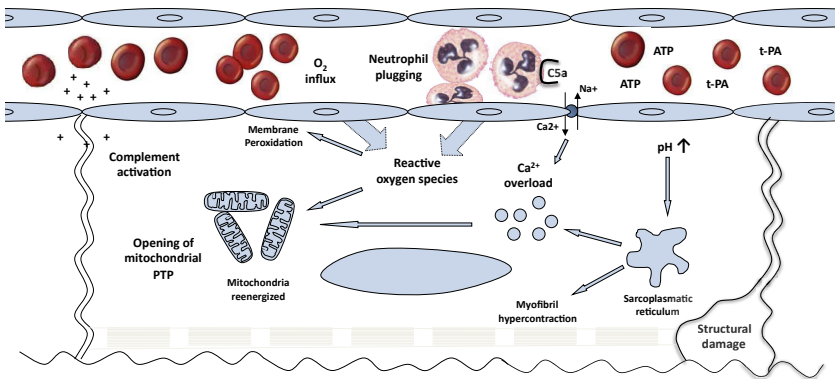

Figure 3: Simplified overview of some factors contributing to reperfusion injury
Re-oxygenation triggers free radical production. Activated complement recruits neutrophils and damage the myocardium directly. Oxidative stress and rapid normalization of pH trigger the opening of the mitochondrial permeability transition pore (MPTP) leading to apoptosis. Hypercontracture arise secondary to calcium overload and lead to structural damage of cytoskeleton and intercellular junctions. Pro-inflammatory tissue plasminogen activator (t-PA) is released from endothelial cells. Extracellular adenosine triphosphate (ATP) is released from erythrocytes leading to an exaggerated reactive hyperemia and direct cardiotoxicity.

Mitochondrial damage appears to arise secondary to the opening of a non-specific pore in the inner mitochondrial membrane called the mitochondrial perme-

ability transition pore (MPTP). The MPTP pore remains closed during ischemia but open during reperfusion and lead to the release of pro-apoptotic factors.[16] In addition, over-activation of Poly(ADP-ribose) polymerase-1 (PARP-1) may impair mitochondrial function and accelerate the production of reactive oxygen metabolites.[17]

Myocyte hypercontracture arise secondary to ischemia related calcium overload combined with reoxygenation and rapid normalization of pH at reperfusion, leading to excessive and uncontrolled contraction. Secondary damage is incurred on cytoskeletal structures and myocytes may tear away from the intercellular junctions of adjacent cells.[18-21]

Oxygen dependent generation of free radicals accelerate at reperfusion and damage the myocytes directly.[22] They also stimulate an inflammatory response.[23]

Complement activation is an early event in cardiac ischemia-reperfusion injury,[24] and the activated complement system can induce tissue damage both directly and in-directly.[25-27] Directly, the C5b-C9 membrane attack complex has cytolytic capacity and has been shown to induce myocardial injury.[28, 29] Complement cascade products also appear to injure the endothelium leading to a vicious circle of vasoconstriction, microvascular hypoperfusion and apoptosis.[30, 31] Indirectly, the activated complement factor C5a stimulates neutrophils at reperfusion by inducing chemotactic migration,[32] aggregation,[33, 34] and release of cytotoxic products such as proteases, elastases and reactive oxygen species that destroy the cell membrane and cause cell death.[33-35] Neutrophils activated by C5a may also contribute to microvascular obstruction by plugging of the microcirculation.[36] Neutrophil activation is also stimulated after plasma membrane pospholipase A2 is activated to form arachidonic acid, an important precursor in the inflammatory pathway.[23] Expression of adhesion molecules allows for adhesion to the vascular wall, and changes in the cytoskeleton increase vascular permeability, giving neutrophils access to vulnerable myocytes.[37, 38]

Tissue plasminogen activator (t-PA) is a protease that initiates endogenous fibrinolysis in the vascular compartment via conversion of plasminogen to plasmin. Ischemia and reperfusion triggers its release,[39] and t-PA has been found to possess pro-inflammatory properties that could contribute to tissue injury.[40, 41] It also increases the release of norepinephrine from sympathetic neurons thereby contributing to cardiac arrythmias,[42] and an unfavourable balance between oxygen demand and availability.[43, 44]

Adenosine triphosphate (ATP) is an intracellular energy-rich compound central to the metabolism. ATP is also an extracellular signalling molecule that is released by erythrocytes in ischemia and reperfusion, and is thought to mediate an exaggerated, possibly harmful, reactive hyperemia during reperfusion via en-

dothelial P2Y2 receptor activation.[45-47] ATP has also been found to have direct cardiotoxic effects.[48] However, its degradation product, adenosine, possess several cardioprotective characteristics.[49]

Background to cardioprotection

The opportunity to specifically treat ongoing ischemic injury to the heart is limited, as reperfusion therapy receives the highest possible priority as soon as the attending physician becomes aware of the condition. Reperfusion injury, on the other hand, would lend itself quite well to specific therapy if such therapy would be available in clinical practice.

A wide range of therapies directed at I/R injury are, and have been, under investigation. A complete overview of these is beyond the scope of this section, but it will give a background to the strategies that have been evaluated within this thesis. These strategies include inhibition of the receptor to the activated complement factor C5a by ADC-1004, degradation of cardiotoxic ATP to cardioprotective adenosine by the enzyme apyrase, and therapeutic hypothermia.

ADC-1004 is a truncated and mutated form of the Chemotaxis inhibitory protein of Staphylococus aureus (CHIPS).[50-52] It was developed using FIND®, a directed in-vitro evolution technology that mimics the natural process of creating protein diversity through recombination.[50] ADC-1004 binds to, but does not activate, the C5a receptor, thereby acting as an effective antagonist.[53] By intervening directly at the C5a receptor, it offers the advantage of exerting its effect on circulating neutrophils, prior to the arrival of the neutrophils at the infarct area. This could be a key to effective anti-neutrophil treatment of myocardial ischemia-reperfusion injury.

Cardiotoxic ATP is degraded to adenosine monophosphate (AMP) by apyrase, also called CD39 or nucleoside triphosphate diphosphohydrolase (NTPdase 1). AMP is converted to adenosine by CD73, also called ecto-5'-nucleotidase.[54, 55] Adenosine has four general cardioprotective modes of action: increased oxygen supply/demand ratio, anti-inflammatory effects, stimulation of angiogenesis and conditioning-related cell-signalling.[49] Apyrase has been shown to reduce infarct size when administered prior to ischemia,[56] but administration according to a clinically applicable protocol has not been investigated.

Therapeutic hypothermia is defined as a controlled reduction in core body temperature to below 35°C, with 33°C as a target. Hypothermia is well documented to reduce infarct size when initiated early during ischemia.[57] The cardioprotective effect increases with lower temperature,[58] but the effect has to be

balanced against the occurrence of side effects, which increase at temperatures below 33°C.[59] The mechanism of effect includes a reduced metabolic demand.[60, 61] However, a reduced oxygen requirement does not fully explain the cardioprotective effect of hypothermia and several additive mechanisms have been suggested.[59, 62] Previous experimental studies have also demonstrated that mild hypothermia increases myocardial contractility in excised heart preparations as well as in the in situ heart.[63-65]

Despite a lack of knowledge regarding the optimal technique of cooling and the therapeutic window of treatment, therapeutic hypothermia was evaluated in two clinical trials for efficacy in ST-elevation myocardial infarction (STEMI); COOL-MI and ICE-IT.[66, 67] Both trials were over-all negative. However, the majority of the patients were not cooled according to the protocol and only a minority had a core body temperature below 35°C at reperfusion. In the subgroup that did reach target temperature, a cardioprotective effect was observed. This lead to the hypothesis that reaching the target temperature prior to reperfusion was necessary for a cardioprotective effect.

We investigated this hypothesis in a porcine model, and showed that hypothermia had a potent cardioprotective effect if target temperature was reached prior to reperfusion, but not if reached after reperfusion, figure 4.[68] We also found cooling with intravenous cold saline in combination with an intravascular cooling catheter to be able to lower the body temperature in a rapid and clinically applicable manner. However, the protocol involved cooling during ischemia, possibly affecting also ischemic injury and not only the reperfusion component of I/R injury. The results could therefore not be taken as decisive evidence for an effect on reperfusion injury.

We also investigated the effect of therapeutic hypothermia in cardiogenic shock in a porcine model, showing that cooling improved hemodynamic- and metabolic variables and reduced acute mortality.[69]

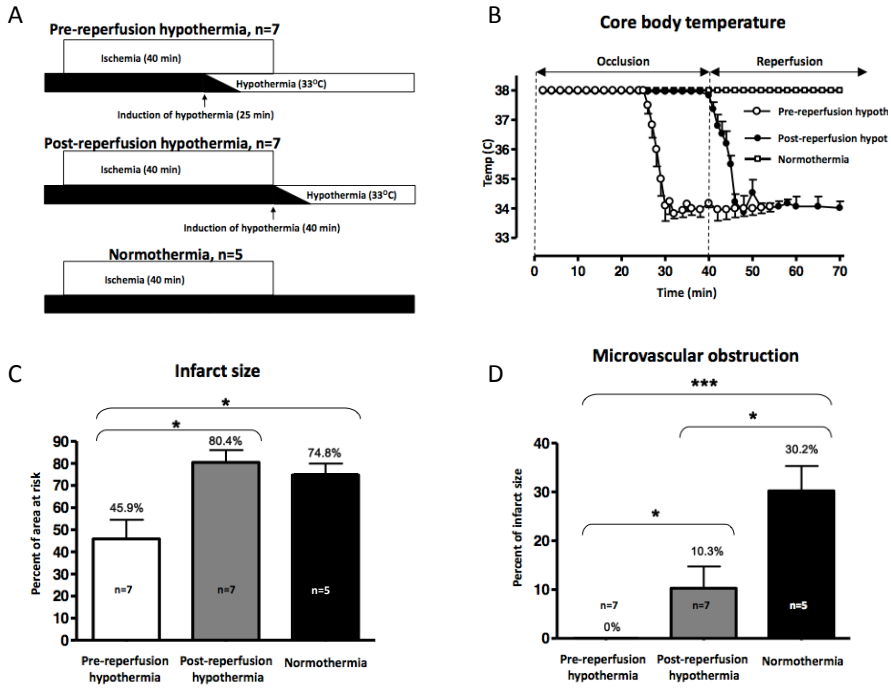

Figure 4: Mild hypothermia reduces infarct size and microvascular obstruction
In a previous experiment we showed that hypothermia had a potent cardioprotective effect if target temperature was reached prior to reperfusion, but not if reached after reperfusion (M Götberg et al, BMC Cardiovasc Disord, 2008;8:7). However, the protocol involved cooling during ischemia, possibly affecting also ischemic injury and not only the reperfusion component of I/R injury. The results could therefore not be taken as decisive evidence for an effect on reperfusion injury.

AIMS

The overall objective of this thesis was to investigate strategies to protect the myocardium against ischemia/reperfusion injury, i.e. cardioprotection.

The specific aims of the included papers were:

- To investigate the cardioprotective effect of the C5a receptor antagonist ADC-1004 (paper I).
- To study the cardioprotective effect of intra-coronary apyrase infusion (paper II).
- To confirm the cardioprotective effect of therapeutic hypothermia (paper III).
- To investigate the effect of the timing and duration of hypothermia treatment on myocardial injury (paper III).
- To study the effect of therapeutic hypothermia on ischemia related coronary t-PA release (paper IV).
- To examine the effect of hypothermia in cardiogenic shock on systemic t-PA release and inflammation (paper V).

MATERIALS AND METHODS

The materials and methods that were used are described below. An overview of the porcine model is presented in figure 5.

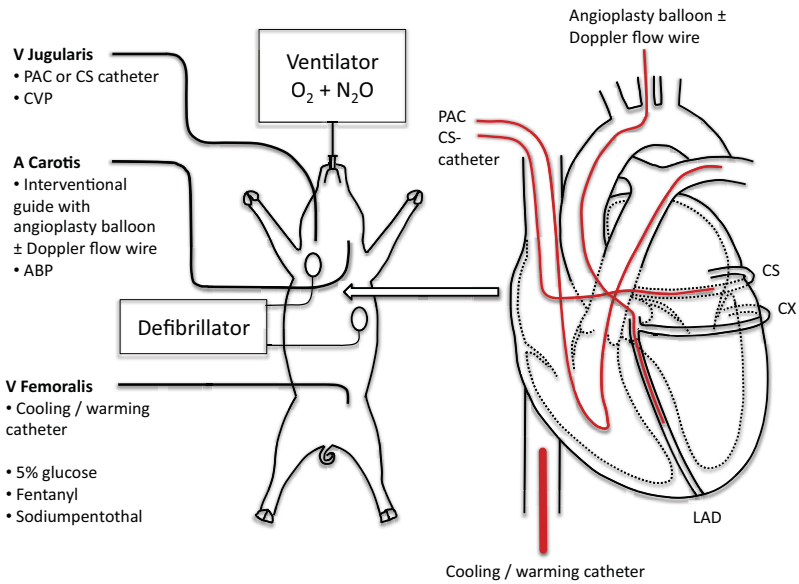

Figure 5: The experimental model
CS = coronary sinus. CX = circumflex artery. LAD = left anterior descending artery. PAC = pulmonary artery catheter. CVP = central venous pressure. ABP = arterial blood pressure. Adapted from AB Ericsson, *Cardioplegia and Cardiac Function,* PhD Thesis, Karolinska Institute, Sweden, 2000.

The porcine model (all papers)

Healthy domestic male and female juvenile pigs weighing 40-50 kg were fasted overnight with free access to water. The animals were premedicated with Ketaminol (Ketamine, Intervet, Danderyd, Sweden; 15 mg/kg) and Rompun (Xylazin, Bayer AG, Leverkusen, Germany; 0.2 mg/kg) intramuscularly 30 min before the procedure. After induction of anesthesia with thiopental (Pentothal,

Abbott, Stockholm, Sweden; 12.5 mg/kg) the animals were orally intubated with cuffed endotracheal tubes. A slow infusion of 1 µl/ml fentanyl (Fentanyl, Pharmalink AB, Stockholm, Sweden) in buffered glucose (25 mg/ml) was started at a rate of 2 ml/min and adjusted if needed. During balanced anaesthesia thiopental (Pentothal, Abbott, Stockholm, Sweden), was titrated towards animal requirements with small bolus doses. Mechanical ventilation was established with a Siemens-Elema 900B ventilator in a volume-controlled mode, adjusted in order to obtain normocapnia (pCO2: 5.0-6.0 kPa). The animals were ventilated with a mixture of nitrous oxide (70%) and oxygen (30%). The pigs were continuously monitored by electrocardiography (ECG). Arterial and central venous blood pressures were measured using MLT0380/D blood pressure transducers (ADInstruments Inc, Colorado Springs, CO, USA). Heparin (200 IU/kg) was given intravenously at the start of the catheterization. A 12 F introducer sheath (Boston Scientific Scimed, Maple Grove, MN, USA) was inserted into the surgically exposed left femoral vein. A 0.021-inch guide wire (Safe-T-J Curved™, Cook Medical Inc, Bloomington, IN, USA) was inserted into the proximal inferior vena cava through the introducer. Using the guide wire, a 10.7 F Celsius Control™ catheter (Innercool Therapies Inc, San Diego, CA, USA) was placed into the inferior vena cava with the tip of the catheter at the level of the diaphragm. Body temperature was measured with a temperature probe (TYCO Healthcare Norden AB, Solna, Sweden) placed in the distal part of the esophagus. The catheter and the temperature probe were connected to the Celsius Control and the system was set to maintain a normal pig body temperature of 38.0 °C. A 6 F introducer sheath (Boston Scientific Scimed, Maple Grove, MN, USA) was inserted into the surgically exposed left carotid artery upon which a 6 F FL4 Wiseguide™ (Boston Scientific Scimed, Maple Grove, MN, USA) was inserted into the left main coronary artery. The catheter was used to place a 0.014-inch PT Choice™ guide wire (Boston Scientific Scimed, Maple Grove, MN, USA) into the distal portion of the LAD. A 3.0-3.5 x 15 mm Maverick monorail™ angioplasty balloon (Boston Scientific Scimed, Maple Grove, MN, USA) was then positioned in the mid portion of the LAD (exact location depending on experimental protocol). A 9 F introducer sheath (Boston Scientific Scimed, Maple Grove, MN, USA) was inserted into the surgically exposed right jugular vein. A 7.5 F CCOmbo™ continuous cardiac output pulmonary artery catheter (Edwards Lifesciences, Irvine, CA, USA) was then inserted into a pulmonary artery. Cardiac output was continuously recorded using a Vigilance™ monitor (Edwards Lifesciences, Irvine, CA, USA). The monitor uses thermal energy emitted by the thermal filament located on the catheter to calculate cardiac output using thermodilution principles. All radiological procedures were performed using an Opescope Pleno™ imaging system (Shimadzu Corp., Kyoto, Japan).

Ischemia was induced by inflation of the angioplasty balloon located in the LAD. An angiogram was performed after inflation of the balloon and before deflation of the balloon in order to verify total occlusion of the coronary vessel and correct balloon positioning. After deflation of the balloon a subsequent angiogram was performed to verify restoration of blood flow in the previously occluded artery.

In animals subject to coronary artery flow measurements, a 0.014-inch, 12 MHz pulsed Doppler flow velocity transducer (Jometrics Flowire, Jomed NV, the Netherlands) was positioned in the LAD, distal to the angioplasty balloon. Continuous coronary velocity flow profiles were displayed and recorded using the Doppler flow wire connected to a FloMap monitor (Cardiometrics, Mountain View, CA, USA). Flow was measured as average peak velocity (APV) in cm/sec.

In animals subject to coronary sinus blood sampling, a short 10 F special catheter of our own design was used to catheterize the Azygos Vein from the introducer placed in the left External Jugular Vein. (In pigs the coronary sinus ends in the Azygos vein.) Then a 6 F MPA coronary catheter (Boston Scientific Scimed, Maple Grove, MN, USA) was passed through the catheter with the tip in the Azygos Vein, into the Coronary Sinus, often with the help of a PT choice guide wire, (Boston Scientific Scimed, Maple Grove, MN, USA).

Imaging (paper I – III)

Infarct size and microvascular obstruction were assessed by ex vivo magnetic resonance imaging (MRI). Area at risk (AAR) was assessed by ex vivo single photon emission computed tomography (SPECT). The MR and SPECT images were analyzed using freely available software (Segment v1.700, Medviso, Lund, Sweden, http://segment.heiberg.se), figure 6.[70, 71]

For MRI, a gadolinium-based contrast agent (Dotarem, *gadoteric acid*, Gothia Medical AB, Billdal, Sweden) was administered intravenously (0.4 mmol/kg) 30 minutes prior to explantation of the heart. The heart was explanted 4 hours after initiation of reperfusion. After explantation, the heart was immediately rinsed in cold saline and the ventricles were filled with balloons containing deuterated water. MRI was performed using a 1.5 T MR scanner (Intera, Philips, Best, the Netherlands). T1-weighted images (repetition time = 20ms, echo time = 3.2ms, flip angle = 70° and 2 averages) with an isotropic resolution of 0.5 mm covering the entire heart were then acquired using a quadrature head coil. Approximately 200 short-axis images were generated of each heart, yielding a high resolution for infarct size delineation. The endocardial and epicardial borders of the left ventricular myocardium were manually delineated in short-axis images.

The volume of the left ventricular myocardium was calculated as the product of the slice thickness (cm) and the area formed by the delineated borders of the epi- and endocardium. The infarct size was determined as the volume of infarcted myocardium (cm^3). The infarct volume was calculated as the product of the slice thickness (cm) and the area of hyperenhanced pixels (cm^2) with a signal intensity above the infarction threshold, defined as >8 SD above the mean intensity of non-affected remote myocardium. Microvascular obstruction was defined as hypointense regions in the core of the infarction which had signal intensity less than the threshold for infarction. The size of the infarct was expressed as percent of the area at risk, in order to adjust for any differences in AAR. The size of microvascular obstruction was expressed as a percent of area at risk or infarct size, depending on the protocol.

SPECT was used to assess the AAR as percent of left ventricular myocardium (LVM). 1000 MBq of ^{99m}Tc-tetrofosmin were administered intravenously 10 minutes before deflation of the angioplasty balloon. Ex vivo imaging was performed with a dual head camera (Skylight, Philips, Best, the Netherlands) at 64 projections (60 s per projection) with a 64 X 64 matrix and a zoom factor of 2.19, yielding a digital resolution of 4.24 X 4.24 X 4.24 mm. Iterative reconstruction using maximum likelihood-expectation maximization (MLEM) was performed with a low-resolution Butterworth filter with a cut-off frequency set to 0.6 of Nyquist and order 5.0. No attenuation or scatter correction was applied. Finally short and long-axis images were reconstructed. The endocardial and epicardial borders of the left ventricle that were manually delineated in the MR images were copied to the co-registered SPECT images. A SPECT defect was defined as a region within the MRI-determined myocardium with counts lower than 55% of the maximum counts in the myocardium.[72]

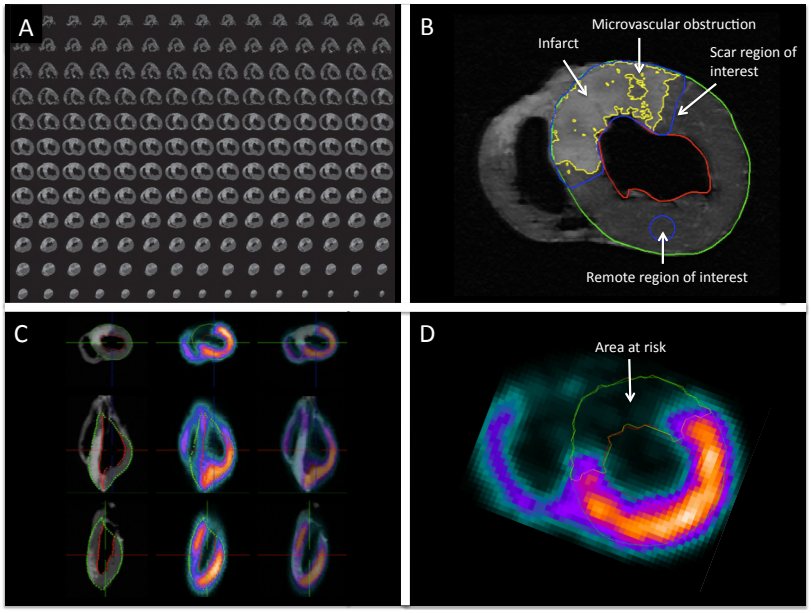

Figure 6: Image analysis
(**A**) Approximately 200 short-axis MR images, each 0.5 mm thick, were analyzed from every heart. (**B**) In each slice the endocardium and the epicardium were manually delineated, as well as the regions of interest for infarct and for remote myocardium. Infarcted myocardium was defined as hyper-enhanced myocardium with signal intensity above 8 SD of the signal intensity in the remote myocardium. Such tissue was automatically delineated along with microvascular obstruction. Microvascular obstruction was defined as hypointense regions in the core of the infarction with signal intensity less than the threshold for infarction. (**C**) The endocardial and epicardial borders of the left ventricle that were delineated in the MR images were copied to co-registered SPECT images, (**D**) from which the area at risk was calculated.

Analysis of blood samples (all papers)

Plasma levels of Troponin T were analysed using the Elecsys immunoassay system (Roche Diagnostics Scandinavia, Bromma, Sweden). The white blood cell count was measured using a Sysmex XE-5000 automated hematology analyzer (Sysmex Corporation, Kobe, Japan). Blood-gases were analyzed in an automated bench top analyzer (Radiometer Medical ApS, Brønshøj, Denmark). Plasma levels of ADC-1004 were analysed by an ELISA method. In this ELISA, the plates were coated with 2 mg/ml of a mouse mab against ADC-1004. The plates were washed and blocked (3% milkpowder in PBS 0.05% Tween 20) and then incubated with plasma samples. ADC-1004 was detected with 3 µg/ml polyclonal rabbit anti-CHIPS N-terminal IgG (IgG produced by immunization of a rab-

bit with a KLH-coupled synthetic peptide corresponding to CHIPS N-terminal amino acids 1-14) and horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Southern Biotech, Birmingham, AL, USA). Plasma concentrations of t-PA were determined by commercial ELISA kits (TintElize t-PA, Biopool AB, Umeå, Sweden; COALIZA PAI, Chromogenix, Haemochrom Diagnostica AB, Mölndal, Sweden; and TriniLIZE t-PA Antigen, Trinity Biotech, Bray, Ireland). All samples from one experiment were assayed in duplicate on the same microtest plate. Levels of secreted TGF-β1, TNF-α, IL-6 and IL-10 were measured by quantitative sandwich enzyme-linked antibody immunoassay technique (R&D System Europe Ltd, Abingdon, UK). Optical density was determined on a microplate reader (Spectramax Plus 384; Molecular Devices Corporation, Sunnyvale, CA, USA).

Calculation and statistics (all papers)

Calculations and statistics were performed using the GraphPad Prism software (version 4.0 and 5.0 depending on paper; GraphPad Software Inc., La Jolla, CA, USA). Values are presented as mean ± SEM. Two-tailed Mann-Whitney's test was performed to test for differences in mean values, except in paper III where Student's t-test was used. Multiple comparisons between variants of cooling protocols were tested with ANOVA. Linear regression was used to test for correlations. Regression analysis was performed post hoc, in an exploratory manner. Statistical significance was accepted when $p < 0.05$.

The net release of t-PA over the vascular bed was calculated as the difference between arterial and venous samples. Total t-PA release was calculated by factoring net t-PA release against changes in blood flow (LAD-flow in paper IV and cardiac output in paper V) compared to baseline (units consisting of (ng/ml – ng/ml) x (l/min / l/min)).

Ethics (all papers)

All experiments conform to the Guide for the Care and Use of Laboratory Animals, US National Institute of Health (NIH Publication No. 85-23, revised 1996) and was approved by the local animal research ethics committee.

EXPERIMENTAL PROTOCOLS AND RESULTS

The protocols for and results of the experiments are presented under the respective subheading. An overview is presented in figure 7.

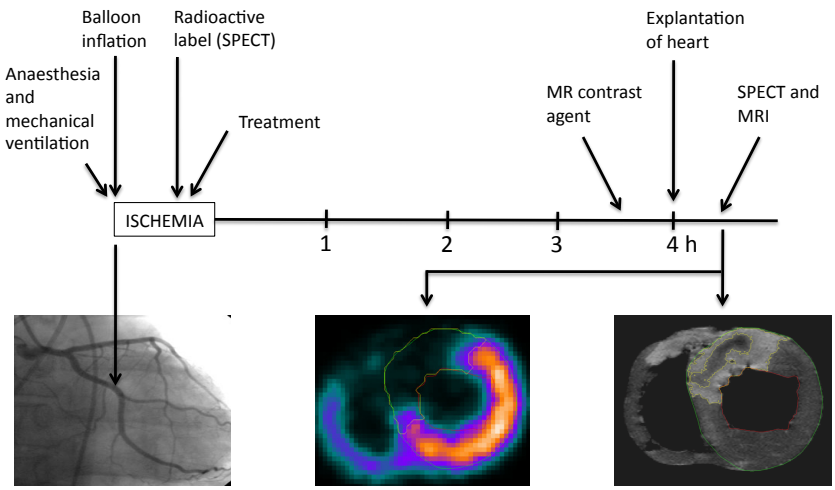

Figure 7: Experimental timeline for paper I through III
Anesthesia and mechanical ventilation was established prior to experimentation. A forty minute episode of ischemia was followed by four hours of reperfusion. The treatment under investigation and SPECT isotope were administered prior to reperfusion. MR contrast agent was administered 30 minutes prior to explantation of the heart. The heart was analyzed ex vivo by SPECT and MRI for infarct size, microvascular obstruction and area at risk.

Paper I:

Treatment with the C5a receptor antagonist ADC-1004 reduces myocardial infarction in a porcine ischemia-reperfusion model

Protocol: The angioplasty balloon was placed in the LAD, immediately distal to the first diagonal branch. Ischemia was induced by inflation of the angioplasty

balloon for 40 minutes. Twenty minutes before balloon deflation the animals were randomized to treatment with an intravenous bolus dose of ADC-1004 (175 mg, n=8) or saline (0.9 mg/ml, n=8). The observers were blinded to the treatment at randomization and analysis.

To monitor the plasma concentration of ADC-1004, plasma samples were collected five minutes after administration, at reperfusion; and one, two, three and four hours after reperfusion. Blood levels of leukocytes and Troponin T, blood gases and hemodynamic parameters were measured according to the protocol specified in the appended article.

Prior to the in-vivo experiment, the potency of ADC-1004 to the C5a receptor was estimated in an in-vitro assay where C5a-induced calcium mobilization was studied by flow cytometry.[53] Pig neutrophils were used and the ability of ADC-1004 to shift C5a-induced concentration-response curves was analysed by Schild-plots, yielding a potency estimate pA2 of 29 nM for ADC-1004 to pig neutrophils. It has been reported that clinically effective concentrations correlates closely with the concentration required for 75% receptor occupancy calculated from the in vitro potency,[73] which in the case of ADC-1004, would give an estimated effective concentration of about 90 nM. Thus, the aim was to give a dose that kept the plasma concentration at or above 90 nM throughout the experiment.

Results: ADC-1004 treatment significantly reduced the primary end-point, infarct size relative to the area at risk, by 21% (ADC-1004: 58.3±3.4 vs control: 74.1±2.9 %AAR, p=0.007) (Figure 8 and 9). Infarct size unadjusted for differences in area at risk was also reduced in the ADC-1004 group, although it did not reach statistical significance (ADC-1004: 23.0±3.0 vs control: 27.1±1.4 %LVM, p=0.16). The extent of microvascular obstruction was similar between the groups (ADC-1004: 2.2±1.2 vs control: 5.3±2.5 %AAR, p=0.23) (Figure 8). A large variation in microvascular obstruction was observed in both groups.

The mean plasma concentration of ADC-1004 at explantation was 83±8 nM, figure 10. The degree of reactive leukocytosis in peripheral venous blood was similar between treatment and control at sacrifice (ADC-1004: 149.5±12.1 vs control: 156.5±15.0 percent of baseline value, NS). Troponin T release mirrored unadjusted infarct size. Blood-gases and hemodynamic parameters were similar between the groups (please see appended article for further information).

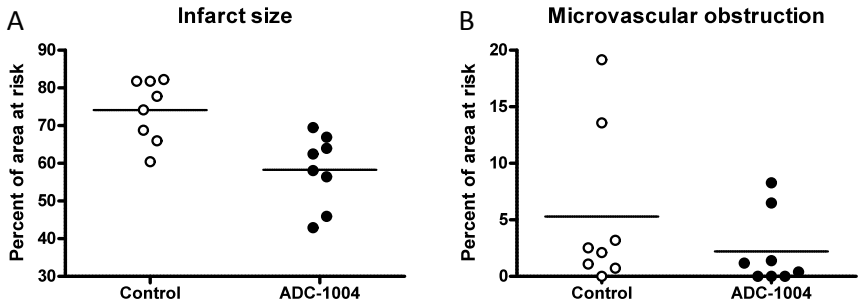

Figure 8: Infarct size and microvascular obstruction
(**A**) ADC-1004 causes a relative reduction in infarct size of 21%, p=0.007. (**B**) Microvascular obstruction was similar between the groups, p=0.23. Horizontal lines denote mean.

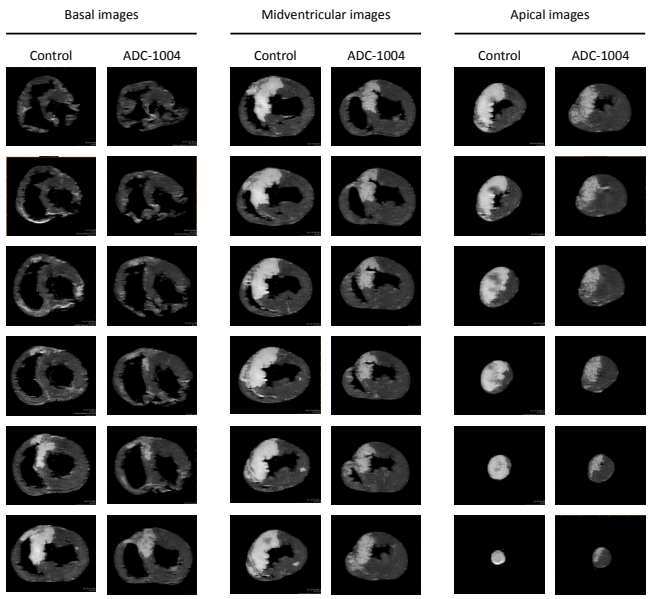

Figure 9: Imaging
Delayed contrast enhanced MR images from one typical animal from each group. Infarcted myocardium (white) is defined as hyper-enhanced myocardium with signal intensity above eight standard deviations of the signal intensity in the remote myocardium. Microvascular obstruction is defined as hypointense regions in the core of the infarction with signal intensity less than the threshold for infarction.

Paper II:

Apyrase treatment of myocardial infarction according to a clinically applicable protocol fails to reduce myocardial injury in a porcine model

Protocol: The angioplasty balloon was placed in the LAD, immediately distal to the first diagonal branch. Ischemia was induced by inflation of the angioplasty balloon for 40 minutes. Twenty minutes before balloon deflation and reperfusion the animals were randomized to 40 min of 1 ml/min intracoronary infusion of apyrase (Sigma-Aldrich, Stockholm, Sweden; 10 U/ml, n = 8) or saline (0.9 mg/ml, n = 8). The infusion was made through an over-the-wire balloon, selectively into the ischemic area.

A separate group of animals was used to verify cardiac effects of the infusion of apyrase. They were fitted with intracoronary flow transducers, and received an intracoronary infusion of apyrase (n=5) as described above during a 10 minute period of ischemia, whereafter reactive coronary hyperemia was measured and compared to controls (n=8).

Results: No differences were observed between the apyrase group and saline group with respect to IS (75.7±4.2 vs 69.4±5.0 %AAR, p=NS) or MO (10.7±4.8 vs 11.4±4.8 %IS, p=NS) (Figure 11). Infarct size unadjusted for differences in area at risk was 28.9±3.1 %LVM in the apyrase group and 27.0±3.8 %LVM in the saline group, p=NS. Hemodynamic parameters and blood gases were similar between the groups throughout the experiment (please see appended article for further information). In the group where coronary blood flow was measured, a pronounced postischemic reactive hyperemia was observed. Infusion of apyrase caused a statistically significant increase in the later part of the hyperemic flow (Figure 11).

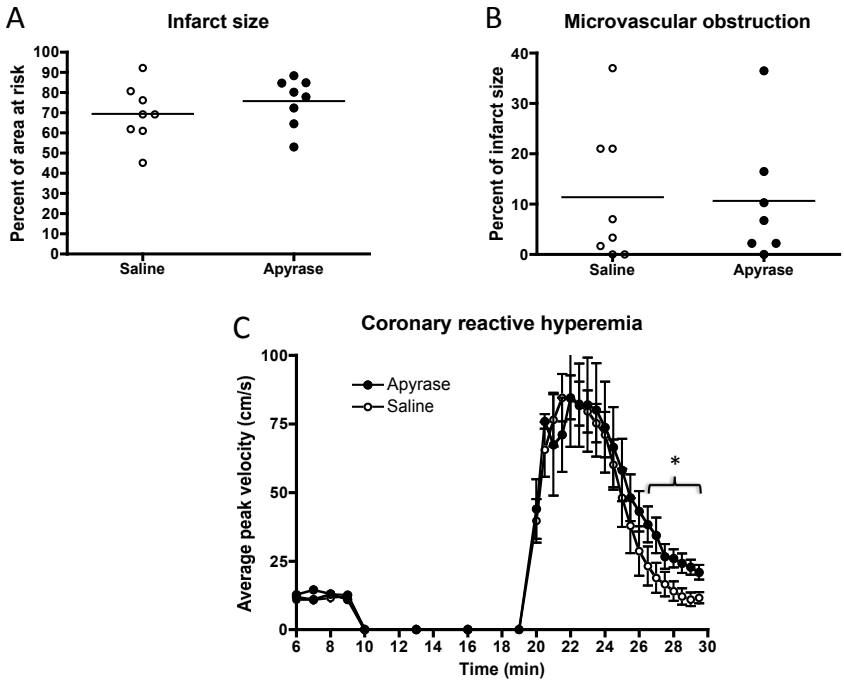

Figure 11: Infarct size, microvascular obstruction and reactive hyperemia
No differences were observed between the groups with respect to (**A**) infarct size or (**B**) microvascular obstruction. Horizontal lines denote mean. (**C**) Post ischemic coronary reactive hyperemia was increased during the later phase by infusion of apyrase. Error-bars denote SEM.

Paper III:

Timing of hypothermia in relation to myocardial reperfusion

Protocol: The protocol for this experiment should be viewed against the background of a previous study where hypothermia was shown to possess potent cardioprotective properties if target temperature was reached prior to reperfusion, but not if reached after reperfusion (Figure 4).[68] However, the protocol involved cooling during ischemia, possibly affecting also ischemic injury and not only the reperfusion component of I/R injury.

Paper III was based on the investigation of the cardioprotective effect of hypothermia in 5 different study groups. Group 1 through 3 were designed to answer the question if hypothermia would specifically reduce reperfusion injury. There-

fore, these groups had an identical duration of *normothermic* ischemia. Cooling was performed during the last five minutes of ischemia in the two treatment groups, and the duration of ischemia was extended with five minutes to a total of 45 minutes. The experimental animals were randomized between group 1, 2 and 3.

1. Normothermia (controls, n=8): 40 minutes of ischemia. Normothermia throughout the experiment.
2. Combination hypothermia (n=8): 45 minutes of ischemia. Cooling with a combination of cold saline infusion (1000 ml, 4°C) and endovascular cooling catheter. Cooling started after 40 minutes of ischemia. Hypothermia was actively maintained with cooling catheter for 30 minutes.
3. Cold saline alone (n=8): 45 minutes of ischemia. Cooling with cold saline infusion (1000 ml, 4°C) alone. Cooling started after 40 minutes of ischemia.

The purpose of group 4 and 5 was to investigate if a longer duration of hypothermia would increase the cardioprotective effect. Experimentation in group 4 was performed time-wise freestanding, and was compared with a retrospective study group from the experiment mentioned above, group 5:

4. Extended hypothermia (n=8): 40 minutes of ischemia. Cooling with a combination of cold saline infusion (1000 ml, 4°C) and endovascular cooling catheter. Cooling started after 25 minutes of ischemia. Hypothermia actively maintained with cooling catheter for 60 minutes after reperfusion.
5. Shorter active hypothermia (n=7): 40 minutes of ischemia. Cooling with a combination of cold saline infusion (1000 ml, 4°C) and endovascular cooling catheter. Cooling started after 25 minutes of ischemia. Hypothermia actively maintained with cooling catheter for 15 minutes after reperfusion.

In all experimental animals, a normal porcine body temperature of 38°C was established prior to induction of ischemia. The angioplasty balloon was placed in the LAD, immediately distal to the first diagonal branch, in all animals. Target temperature was 33°C and successful cooling was defined as a core body temperature below 35°C.

Results: Measurements of core body temperature during the experiment are shown in figures 12-14. A detailed statistical report is available in the appended article. Briefly, all hypothermic groups apart from the one cooled with cold saline alone reached a temperature of <35°C at the time of reperfusion. There was no difference between extended and shorter active hypothermia at the onset of reperfusion, but there was a difference during the later phase of reperfusion.

Combination hypothermia reduced the infarct size by 18 % compared to normothermic controls (60.8±4.9 vs 73.8±4.0 %AAR; p=0.03) (Figure 12). Combination hypothermia also reduced the size of microvascular obstruction (0.5±0.5 vs 21.5±5.2 %IS; p<0.001). Please see the appended article for information regarding hemodynamic variables.

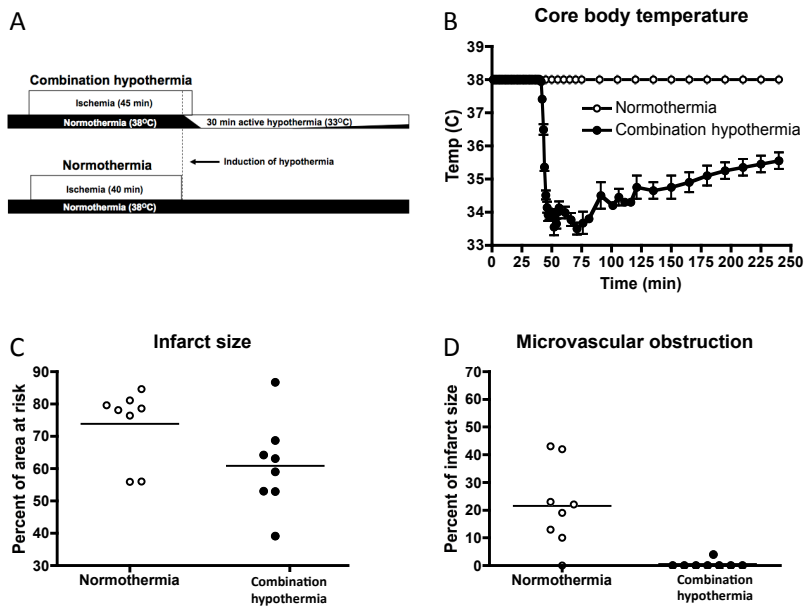

Figure 12: Results of combination hypothermia
(**A**) Hypothermia was started after 40 min of ischemia by an infusion of saline combined with an endovascular cooling catheter. The duration of ischemia was prolonged by 5 min in order to achieve the same normothermic duration of ischemia. (**B**) Combination hypothermia resulted in a rapid reduction in core body temperature to below 35º C in approximately 5 min. Hypothermia was maintained for 30 minutes followed by passive re-warming. After active cooling was discontinued, core body temperature increased by approximately 0.5-0.8ºC/h. Error-bars represent SEM. (**C**) Combination hypothermia caused an 18% relative reduction in infarct size compared to normothermia despite longer total ischemic time (p=0.03). (**D**) Combination hypothermia virtually eliminated microvascular obstruction (p<0.001). Horizontal lines denote mean

Cold saline alone reduced the extent of microvascular obstruction (5.5±2.5 vs 21.5±5.2 %IS, p<0.05, cold saline hypothermia vs normothermia) but not the infarct size (73.0±4.3 vs 73.8±4.0 %AAR, p>0.05, cold saline hypothermia vs normothermia) (Figure 13). The extent of microvascular obstruction did not differ significantly compared to combination hypothermia (0.5±0.5 %IS, p>0.05)

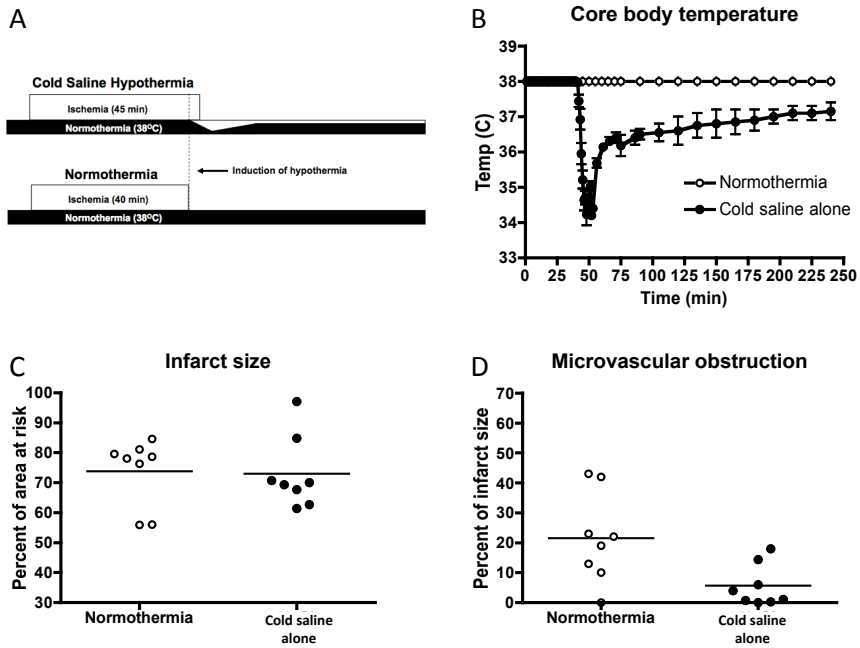

Figure 13 Results of cold saline alone
(**A**) Hypothermia was started after 40 min of ischemia by a single infusion of 1000 ml of cold saline solution. The duration of ischemia was prolonged by 5 min in order to achieve the same duration of normothermic ischemia. (**B**) Cold saline alone failed to achieve a body temperature below 35ºC at reperfusion. Error-bars represent SEM. (**C**) Cold saline failed to reduce infarct size ($p>0.05$), (**D**) but reduced microvascular obstruction compared to normothermia ($p<0.05$). Horizontal lines denote mean.

The infarct size was equally reduced in the group with extended hypothermia compared to the group with shorter active hypothermia (48±7 vs 46±8 %AAR, $p>0.05$, extended vs shorter active hypothermia). Extended hypothermia reduced microvascular obstruction to the same degree that shorter active hypothermia did (0.2±0.2 vs 0 %IS, $p>0.05$, extended vs shorter active hypothermia) (Figure 14).

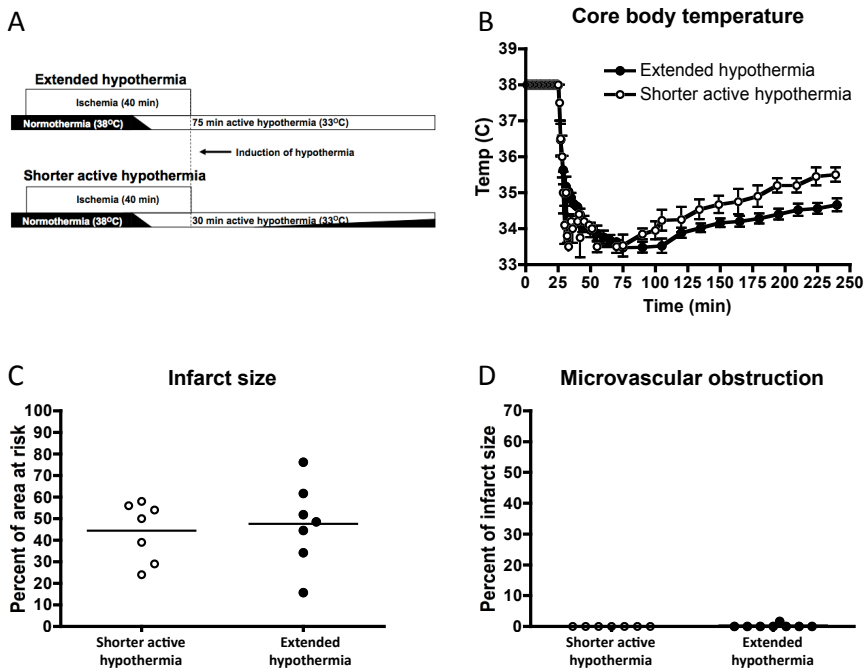

Figure 14: Results of extended hypothermia
(**A**) Hypothermia was maintained for 60 minutes after the onset of reperfusion in the extended hypothermia group, compared to 15 minutes in the group with shorter active hypothermia. (**B**) Extended hypothermia resulted in a lower core body temperature during the later phase of reperfusion compared to shorter active hypothermia. Error bars represent SEM. (**C**) Infarct size and (**D**) microvascular obstruction were similar between the groups. In one pig in the extended hypothermia group, SPECT data were lost. The data regarding infarct size from that pig were excluded but data regarding microvascular obstruction were included in the analysis. Horizontal lines denote mean.

Paper IV:

Mild hypothermia markedly reduces ischemia related coronary t-PA release

Protocol: In all experimental animals, a baseline temperature of 37°C was established and maintained for 30 minutes. The pigs were then randomized to hypothermia (n=8) or control (n=8). The pigs randomized to hypothermia were cooled with the endovascular cooling catheter to a temperature of 34.0°C, prior to balloon inflation, which was then maintained until sacrifice. The pigs ran-

domized to the control group were maintained at 37°C until sacrifice. The angioplasty balloon was placed in the LAD, immediately distal to the first diagonal branch. Ischemia was induced by inflation of the angioplasty balloon for 10 minutes.

t-PA was measured at baseline, one minute before reperfusion (=9 min of ischemia); and one, five and 10 minutes after reperfusion. Samples were collected simultaneously from a peripheral artery and in the venous blood from the coronary sinus. Blood pressure, heart rate and coronary artery flow in the LAD was measured continuously.

Results: There were no significant differences in basal t-PA levels in peripheral arterial or coronary sinus samples. Net t-PA release increased during reperfusion. Hypothermia inhibited the increase in net t-PA release during reperfusion (peak value 9.44±4.34 ng/ml vs 0.79±0.45 ng/ml, p=0.02). The effect was even more prominent when an estimation of total t-PA release was performed (factorial correction for blood flow, see materials and methods section) with mean peak value 26 fold higher in the control group than in the hypothermia group (69.74±33.86 units vs 2.62±1.10 units, p=0.01), figure 15.

Coronary blood flow in the LAD increased dramatically during the early reperfusion phase (previously published data).[46] The peak flow observed during post ischemic reactive hyperemia was reduced by 43% in the hypothermia group compared to the control group (p<0.01). Peak flow occurred 2.5 min after reperfusion and was 83,6±7.8 cm/s in the normothermic group and 50,6±7.2 cm/s in the hypothermic group. There was no observed difference in coronary flow between the groups during baseline or 7 minutes after reperfusion. Please see the appended article for hemodynamic and blood-gas data.

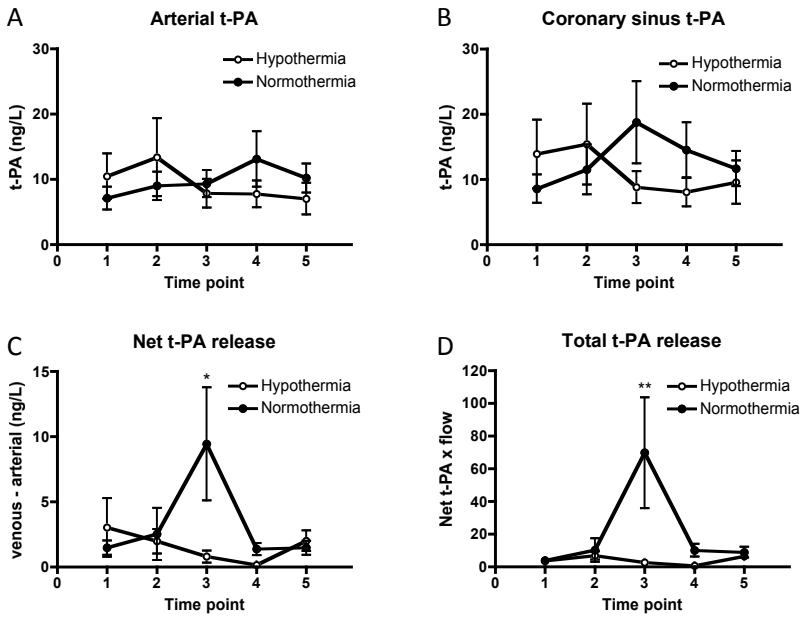

Figure 15: Basal t-PA levels and t-PA release
There were no statistically significant differences in basal t-PA levels in (**A**) peripheral arterial or (**B**) coronary sinus samples. (**C**) Net t-PA release over the coronary bed was inhibited my mild hypothermia during reperfusion. (**D**) The effect was even more prominent when an estimation of total t-PA release was performed. Error-bars represent SEM.

Paper V:

Hypothermia in cardiogenic shock reduces systemic t-PA release

Protocol: The primary objective of this study was to investigate the effect of therapeutic hypothermia in cardiogenic shock, on the basal levels of t-PA and on the release of t-PA from the peripheral vascular bed. The secondary objective was to investigate a possible effect on inflammation, as measured by the pro-inflammatory cytokines interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α); and the anti-inflammatory cytokines interleukin 10 (IL-10) and transforming growth factor beta 1 (TGF-β1).

Cardiogenic shock was induced by inflation of the angioplasty balloon in the proximal LAD for 40 min, followed by 110 minutes of reperfusion (Figure 16). Immediately before reperfusion the pigs were randomized to hypothermia (n=8) or to normothermia (n=8). Hypothermia was induced and maintained using

the endovascular cooling system alone. After reaching the target temperature of 33°C, hypothermia was maintained throughout the experiment. In the normothermic group, the endovascular catheter was used to maintain a normal pig body temperature of 38° C.

Plasma samples were collected from the carotid artery and the inferior vena cava for analysis of t-PA at baseline and every 30 minutes until the experiment ended. Venous plasma samples were collected for analysis of IL-6, IL-10, TNF-α and TGF-β1 at baseline and at the end of the experiment. Blood gases were analyzed every 30 minutes throughout the experiment. The blood gas values were corrected for core body temperature at the time that the samples were taken from the animals.

Results: Measurements of core body temperature are shown in figure 16. At the time of initiation of ischemia and at reperfusion there was no difference in temperature between the groups. Hypothermia was induced at the time of reperfusion by the endovascular catheter. 45 minutes after initiation of hypothermia treatment, the mean temperature in the hypothermia group was 33.6 ± 0.7° C. The mean cooling rate was 5.9 ° C/h.

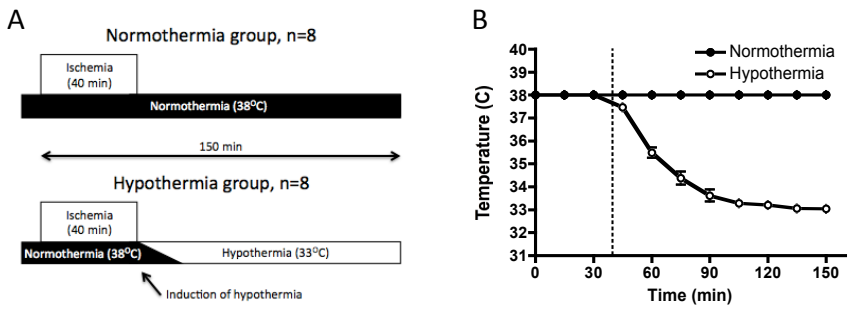

Figure 16: Experimental protocol and temperature data
(**A**) Experimental protocol. (**B**) Core body temperature in the two groups. The dashed line illustrates the time of randomization and initiation of hypothermia. Error-bars denote SEM.

Detailed hemodynamic and blood-gas data are shown in figure 17 and table 1. Briefly, hypothermia resulted in an increased mean arterial pressure. Heart rate was decreased but stroke volume increased leading to a slightly increased cardiac output in the hypothermic group. Mixed venous saturation, arterial pH and base excess were higher in the hypothermic group. All these differences were statistically significant.

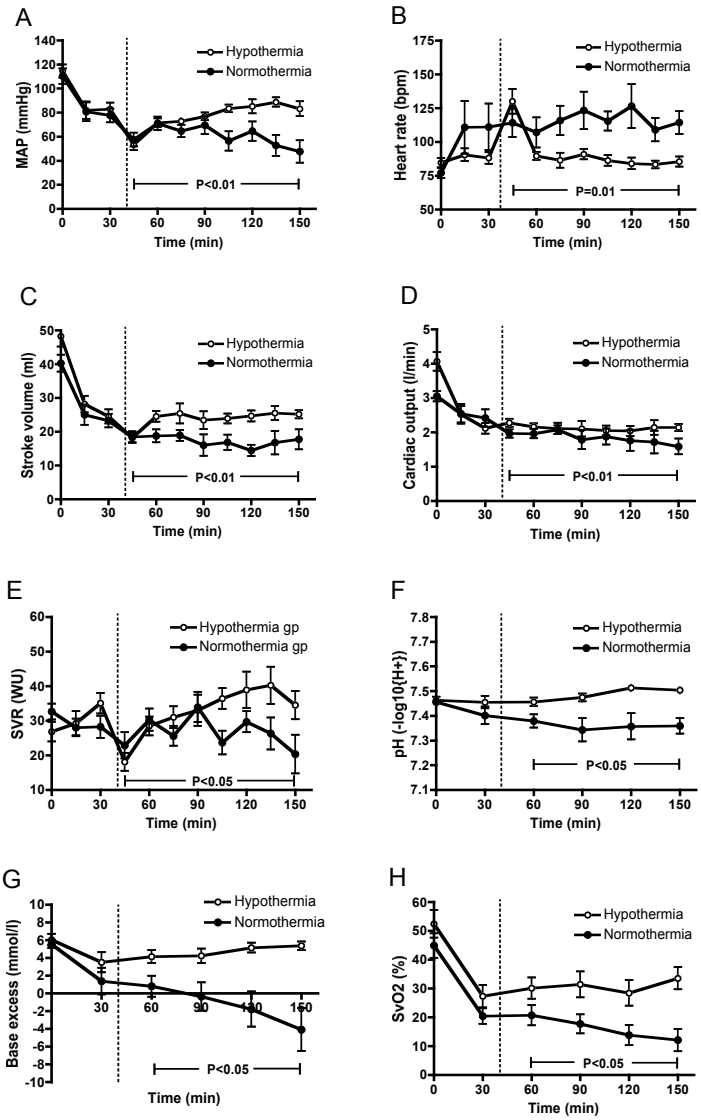

Figure 17: Hemodynamic and blood-gas data
Hypothermia increased (**A**) mean arterial pressure and decreased (**B**) heart rate. Due to a higher (**C**) stroke volume, hypothermia slightly increased (**D**) cardiac output. (**E**) Systemic vascular resistance was also increased in the hypothermic group. (**F**) Arterial pH and (**G**) base excess were higher, indicating no development of metabolic acidosis in the hypothermia group. (**H**) Mixed venous saturation was higher in the hypothermia group indicating lower metabolic demand in peripheral tissues. Error-bars denote SEM.

Table 1: Mean values from randomization until the end of the experiment

Variable	Hypothermia (n=8)	Normothermia (n=8)	P-value
Mean arterial pressure (mmHg)	77 ± 4	60 ± 3	$p < 0.01$
Stroke volume (ml)	23.8 ± 0.9	17.2 ± 0.5	$p < 0.01$
Heart rate (bpm)	92 ± 6	116 ± 3	$p = 0.01$
Cardiac output (l/min)	2.1 ± 0.03	1.8 ± 0.06	$p < 0.01$
Systemic vascular resistance (WU)	32.6 ± 2.5	26.6 ± 1.6	$p < 0.05$
SvO_2 (%)	30 ± 1	16 ± 2	$p < 0.05$
Arterial pH ($\log_{10}\{H^+\}$)	7.49 ± 0.01	7.36 ± 0.01	$p < 0.05$
Base excess (mmol/l)	4.7 ± 0.3	-1.4 ± 1.0	$p < 0.05$
Venous t-PA (ng/ml)	2.11 ± 0.08	7.30 ± 1.93	$p < 0.05$
Arterial t-PA (ng/ml)	1.90 ± 0.08	4.70 ± 0.90	$p < 0.05$
Net t-PA release (ng/ml)	0.60 ± 0.12	2.16 ± 1.09	$p < 0.05$
Total t-PA release (units)	0.12 ± 0.06	1.79 ± 0.78	$p < 0.05$

Net t-PA release was calculated as the difference between arterial samples and venous samples. Total t-PA release was calculated by factoring net t-PA release against changes in cardiac output compared to baseline. WU = Wood units. SvO2 = mixed venous saturation. Data are presented as mean ± SEM.

Detailed information on t-PA is given in table 1 and figure 18. In brief, cardiogenic shock resulted in increased basal levels of venous and arterial t-PA, as well as in net- and total t-PA release (factorial correction for blood flow, see materials and methods section). Hypothermia inhibited any increase in t-PA, both in basal levels and in net- and total release. All these differences were statistically significant.

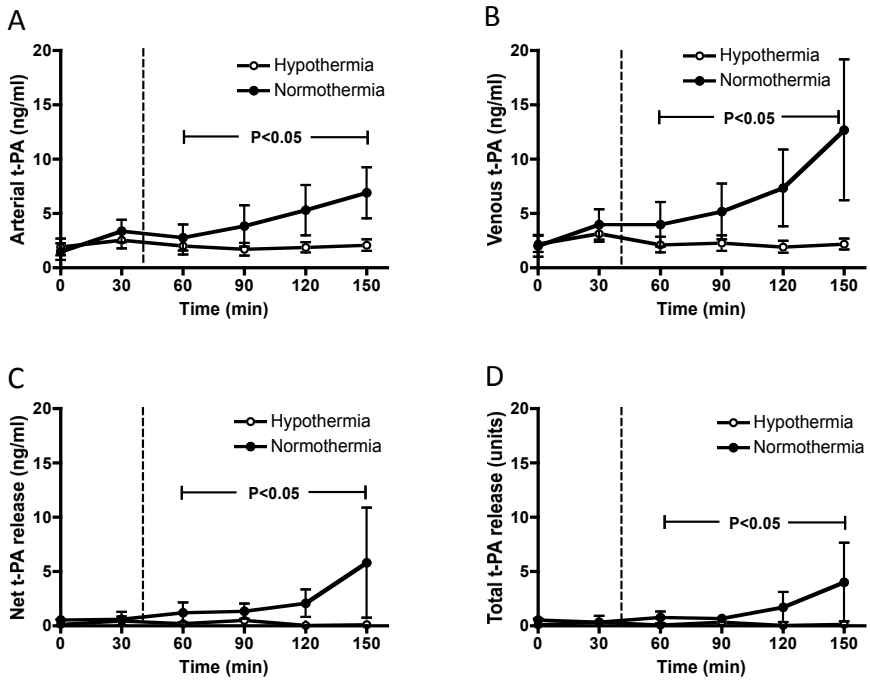

Figure 18: t-PA data
Hypothermia inhibited the increase in (**A**) arterial and (**B**) venous t-PA levels that cardiogenic shock induced. It also inhibited the increase in (**C**) net- and (**D**) total t-PA release. Net t-PA release was calculated as the difference between arterial samples and venous samples. Total t-PA release was calculated by factoring net t-PA release against changes in cardiac output compared to baseline. Error-bars denote SEM.

There was a statistically significant inverse correlation between arterial baseline and end-experimental levels of t-PA; and mean arterial pressure, systemic vascular resistance, stroke volume, pH and mixed venous oxygen saturation. There was a strong trend towards statistical significance for an inverse correlation between arterial t-PA and cardiac output. A statistically significant positive correlation was observed for heart rate (Figure 19). The results for arterial values were valid also for venous values, apart from cardiac output, heart rate (both clearly non-significant) and stroke volume (trend towards significance). For further information, please see the appended article. Subgroup analysis revealed that the correlations were dependent on observations in the normothermic group (table 2 and 3).

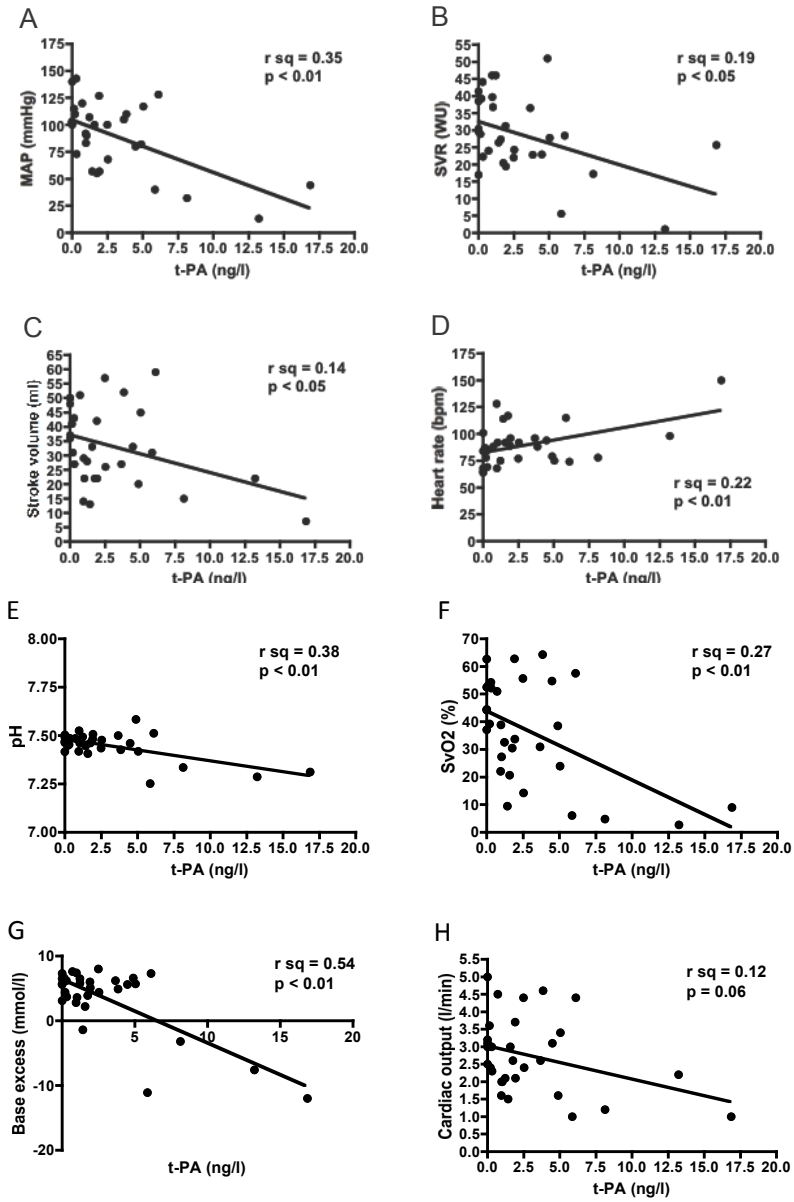

Figure 19: Regression analysis

By linear regression analysis, the arterial baseline and end-experimental levels of t-PA were found to correlate with (**A**) mean arterial pressure, (**B**) systemic vascular resistance, (**C**) stroke volume, (**D**) heart rate, (**E**) pH, (**F**) mixed venous oxygen saturation and (**G**) base excess. There was a trend towards statistical significance for (**H**) cardiac output.

Table 2: Correlations between t-PA and metabolic parameters

	pH		SvO2		BE	
	r^2	p	r^2	p	r^2	p
Normothermia, arterial t-PA	0.60	<0.01	0.40	0.01	0.65	<0.01
Hypothermia, arterial t-PA	0.31	NS	0.01	NS	0.65	NS
Normothermia, venous t-PA	0.43	<0.01	0.28	0.03	0.40	0.01
Hypothermia, venous t-PA	<0.01	NS	0.02	NS	<0.01	NS

Statistically significant correlations were observed between t-PA levels and pH, BE (base excess) and SvO2 (mixed venous saturation), in the normothermic group. The unit for pH is –log10{H+}. The linear regression analysis is based on the baseline- and end experimental values.

Table 3: Correlations between t-PA and hemodynamic parameters

	MAP		HR		SV		CO		SVR	
	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p
Normothermia, arterial t-PA	0.50	<0.01	0.27	0.05	0.29	<0.05	0.25	0.06	0.39	0.01
Hypothermia, arterial t-PA	<0.01	NS	0.02	NS	0.01	NS	0.03	NS	0.02	NS
Normothermia, venous t-PA	0.43	<0.01	0.07	NS	0.11	NS	0.05	NS	0.47	<0.01
Hypothermia, venous t-PA	<0.01	NS	<0.01	NS	<0.01	NS	<0.01	NS	0.02	NS

Statistically significant correlations were observed between t-PA and most hemodynamic parameters in the normothermic group. MAP = mean arterial pressure. HR = heart rate. SV = stroke volume. CO = cardiac output. SVR = systemic vascular resistance. The linear regression analysis is based on the baseline- and end experimental values.

Cardiogenic shock triggered a statistically significant increase in TGF-β1 and IL-6 but not in TNF-∝ or IL-10 at 150 minutes (Figure 20). However, there were no statistically significant differences between the hypothermic and normothermic groups in any of the measured cytokines (Figure 20); IL-6 (1758±534 vs 3184±1953 %baseline, hypothermia vs normothermia, NS), IL-10 (98±41 vs 132±58 %baseline, hypothermia vs normothermia, NS), TNF-∝ (78±30 vs 77±24 %baseline, hypothermia vs normothermia, NS) and TGF-β1 (1063±418 vs 472±251 %baseline, hypothermia vs normothermia, NS).

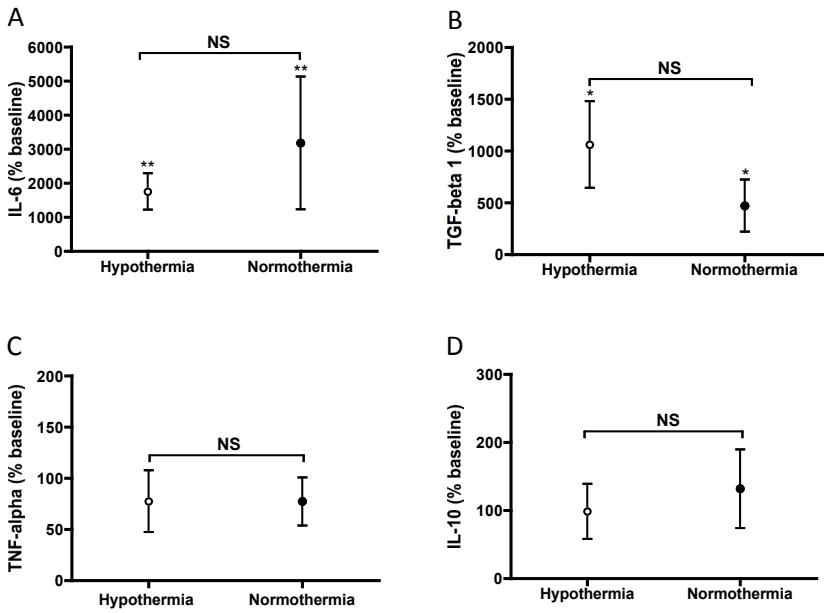

Figure 20: Inflammatory markers
Cardiogenic shock was found to trigger a statistically significant increase in (**A**) IL-6 and (**B**) TGF-β1 but not in (**C**) TNF-∝ or (**D**) IL-10 at 150 minutes. There were no statistically significant differences between the hypothermic and normothermic groups. Please note that the scale on the y-axis differ between the graphs. Error-bars denote SEM.

DISCUSSION

The model

The porcine model was chosen because it is a large animal model that resembles the human physiology, and also allows for a closed chest model utilising human coronary interventional devices. A percutaneous catheter-based approach allows for induction of ischemia with minimum trauma, operation-induced stress and secondary changes in circulatory physiology. The model also offers a possibility of SPECT and MRI for ischemia and infarct size evaluation. Ex vivo MRI allows for acquisition of high-resolution images and objective semiautomatic quantification of myocardial infarction, and correlates closely to histology.[74, 75] MRI and SPECT are also the gold standard methods of evaluation of ischemia and infarct size in clinical practice. Temperature is known to be a major determinant of infarct size.[76] In order to eliminate spontaneous hypothermia as a confounding factor for infarct size development, a normal core body temperature of pigs (38 °C) was maintained during ischemia, or until hypothermia treatment started. The 40 minute duration of ischemia is shorter than in the typical patient with myocardial infarction, who often experience 2-4 hours of ischemia before start of treatment. However, pig infarct progress has been shown to be approximately 7 times more rapid than human,[77] suggesting that the current model represents a human STEMI with approximately five hours duration. A longer duration of ischemia would have resulted in a large established infarct before initiation of treatment.

Cardioprotection by complement inhibition

Paper I evaluated the cardioprotective effect of ADC-1004 treatment, according to a clinically applicable protocol for ST-segment elevation myocardial infarction (STEMI).

Treatment with ADC-1004 was found to significantly reduce infarct size by 21%. The extent of microvascular obstruction was similar between the groups, suggesting that the relative contribution of activated neutrophils to the develop-

ment of microvascular obstruction is less than the contribution of neutrophils to infarct development. As the mean plasma concentration of ADC-1004 four hours after reperfusion was found to be 83 nM, the aim to keep the plasma concentration at a therapeutic level (i.e. at or above 90 nM) was met for the major part of reperfusion.[73] Thus, as shown in the dose prediction experiment described in the experimental protocol section, the circulating neutrophils were blocked at the C5a receptor in the ADC-1004 group and rendered resistant to C5a related activation. The activation has been shown to peak two hours after reperfusion and to rapidly decline thereafter.[78] C5a has also been shown to have a short half-life, with effects resolving within a few minutes.[79] Consequently, a bolus administration of ADC-1004 prior to reperfusion seems to cover the entire therapeutic window for inhibition of C5a-related neutrophil activation. A major advantage with ADC-1004 is that, if it is administered before reperfusion, it will inhibit the circulating neutrophils before they reach the ischemic zone, thereby avoiding even a brief period of activation. There were no significant differences in hemodynamic parameters between the groups, suggesting that ADC-1004 is safe for administration in patients with acute myocardial infarction.

Several other preclinical studies have evaluated the effect of blocking the C5a component, either by blocking the conversion of C5 to C5a, by neutralising antibodies to C5a or by antagonists of the C5a receptor, over all with findings of cardioprotective effects in animal models.[80-83] Exposure of C5a at a sublytic dose prior to ischemia has also been shown to induce cardioprotection,[84] possibly by triggering a preconditioning effect. The preclinical evidence by large, clearly supports a therapeutic possibility in inhibiting C5a related neutrophil activation, which is also in line with the findings in this study.

A few substances have been clinically evaluated recently. The FIRE trial evaluated the effect of inhibition of the neutrophils on infarct size by FX06, a VE-cadherin inhibitor, with the finding of a 58% reduction in necrotic core zone after five days.[85] However, this finding did not remain after four months.[85] The APEX AMI trial evaluated the humanized monoclonal antibody pexelizumab, that binds the C5 component of complement, as an adjunct to PCI in improving 30-day mortality from STEMI.[86] In this trial, mortality was unaffected by pexelizumab treatment. Furthermore, even though pexelizumab had been shown to reduce apoptosis and leukocyte infiltration resulting in reduced myocardial injury in an animal model,[83, 87] pexelizumab treatment initiated prior to reperfusion failed to favourably affect infarct size in a phase 2 trial.[88] However, an antibody to C5 can neither inhibit the C5a that has already been generated nor can it exert its effect prior to the arrival of the substance in the infarct area. A C5a receptor antagonist, on the other hand, offers the advantage of blocking the receptor on the neutrophils prior to the arrival of the neutrophil in the infarct area.

The C5a receptor is also found on cardiomyocytes, and this receptor activates an intracellular signalling cascade involving protein kinase C isoenzyme delta (PKC-δ) that may contribute to ischemia/reperfusion injury.[89] The substance KAI-9803 is a PKC-δ inhibitor that has been shown to reduce myocardial injury in animal models,[90, 91] and is now in clinical development.[92] Effects of ADC-1004 on cardiomyocytes could mediate some of the cardioprotective effect by reducing PKC-δ activation, but this needs to be confirmed in future experiments.

Cardioprotection by apyrase

Paper II was specifically aimed at evaluating the clinical applicability of apyrase treatment in the setting of STEMI. Consequently, apyrase was administered only during the final 20 minutes of ischemia and continued after reperfusion, in order to mimic the clinical situation. The main findings were that infarct size and microvascular obstruction were unaffected by apyrase treatment. However, reactive hyperemia was prolonged.

Previously, apyrase treatment has been shown to reduce infarct size with 43% when administered intravenously 30 minutes prior to a period of 60 minutes of ischemia in a rodent model.[56] In that experiment, 80 U/kg of apyrase was administered in wild-type mice. With an average mouse weight of 20g and a percentage distribution of cardiac output with 18% going to the heart during rest,[93] 0.28 U of apyrase would have been delivered to the heart. In our study, an approximately 1400 times higher dose of 400 U was infused directly into the ischemic area in order to maximize the possible cardioprotective effect of apyrase treatment at this later timepoint.

The finding of prolonged coronary reactive hyperemia induced by apyrase-infusion, suggest that adenosine is generated by the apyrase-infusion. Adenosine has been shown to mediate the later phase of hyperemic flow via A2A receptors on smooth muscle cells.[94, 95] Thus, an increased level of adenosine would be expected to increase the later phase of hyperemia, which it did in our experiment.

The lack of cardioprotective effect of the apyrase-infusion could be explained by species-related differences in the pharmacology of adenosine receptors. It is also possible that apyrase was not able to generate enough adenosine to activate signalling via the A2B receptor, which otherwise has been able to yield cardioprotection when stimulated prior to reperfusion by the receptor agonist AMP 579 in a porcine model.[96] Indeed, the A2B receptor is known to be of lower affinity for adenosine than the A2A receptor,[97] which seemed to respond with the increased flow seen at the end of reactive hyperemia in the apyrase

group.[94, 95] However, there is also evidence of A2A mediated cardioprotection against ischemia-reperfusion injury.[98] Theoretically, the high dose of apyrase that was used could also have a cardiotoxic effect. However, we are not aware of any such evidence in the literature. The lack of effect could also be explained by a species-related imbalance between apyrase and downstream adenosine generation by CD73 as well as by a low release of native ATP and AMP. A relative deficiency of AMP degradation capacity would be in agreement with the uncertain effect of postconditioning in pigs. Two studies of postconditioning in pig have been carried out where one was negative and the other was negative in one arm with 4 cycles of ischemia/reperfusion and positive in another arm with 8 cycles.[99, 100] In another study in a porcine model, preconditioning was found to be cardioprotective.[101] In that study, cardiac adenosine levels were measured in the preconditioning group and found to be increased during preconditioning but attenuated during ischemia. In the control group, the adenosine concentration was elevated during ischemia and low prior to ischemia.[101] The increased adenosine level during ischemia seen in the control group is in agreement with the experimental protocol in our study. This clearly supports the conclusion that the apyrase infusion must be initiated prior to the induction of ischemia in order to generate a cardioprotective effect. It is possible that apyrase treatment could prove to be cardioprotective if initiated earlier than in the current study, but it would be of little interest in clinical practice. These findings may also imply that other tentative triggers of pre- and postconditioning, such as bradykinin, opioids, nitric oxide and reactive oxygen species,[102-104] could be of greater importance than adenosine in conditioning related cardioprotection.

In paper II, vegetable-extracted apyrase was used. Paper II was followed by a small pilot experiment where 0,5 mg/kg of recombinant (human) apyrase or saline was administered intravenously in an otherwise unchanged protocol (3 animals in each group, unpublished data). This experiment confirmed the lack of effect.

Technique, timing and cardioprotective effect of hypothermia

Paper III demonstrated that hypothermia induced before reperfusion despite five minutes of prolonged ischemia reduces myocardial infarct size in pigs by 18 % compared to normothermia and essentially abolishes microvascular obstruction.

A previous experimental study demonstrated the time dependency of the protective effects of hypothermia, with progressively more pronounced effect the ear-

lier hypothermia was initiated after the induction of ischemia.[57] Previous studies have also demonstrated that hypothermia initiated after the onset of reperfusion does not reduce infarct size.[68, 105] Assuming a similarity between this experimental study and the clinical situation, initiation of hypothermia in the catheterization laboratory in a STEMI patient would result in a decrease in infarct size if target temperature was achieved before reperfusion. The cardioprotective effect could be further enhanced if hypothermia was initiated at an earlier stage, for example in the ambulance or in the emergency department.

In this and a previous study from our lab, we demonstrate the efficiency of an infusion of cold saline combined with endovascular cooling to achieve target temperature within five minutes in 40-50 kg pigs.[68] In order for hypothermia to achieve a reduction in infarct size, post-hoc analysis of the ICE-IT and COOL-MI studies indicated that the body temperature needs to be <35 ºC before reperfusion.[66, 67] A problem with using external or endovascular cooling alone is the slow onset of effect. Combination hypothermia appears to provide an approach to rapid cooling with potential clinical applicability.

The current experiment also showed that hypothermia drastically reduces microvascular obstruction. Microvascular obstruction is otherwise prevalent in large myocardial infarctions and the extent of microvascular obstruction is correlated to the size of the infarct.[106] Furthermore, the presence of MO is independently associated with an impaired recovery of left ventricular function and a poor clinical long term outcome.[9, 10] Hypothermia initiated during ischemia reduces MO to a greater extent than infarct size.[68, 107] If hypothermia is initiated at the onset of reperfusion, no reduction in infarct size is observed, however, MO is significantly reduced.[68] These findings differentiate the evolution of infarct and of MO as separate mechanisms and links MO to the injury incurred during reperfusion. They also suggest that it is possible to reduce the extent of microvascular obstruction with therapeutic hypothermia. However, it still needs to be proven that a reduction in microvascular obstruction results in a clinical benefit to the patient.

We also speculated that cold saline alone could reduce infarct size and microvascular obstruction. However, cold saline alone did not reduce infarct size. The lack of effect in infarct reduction is probably due to failure to reach target temperature at the time of reperfusion, and for being unable to sustain hypothermia during the early phase of reperfusion. Cold saline alone did, however, reduce MO with 75% compared to normothermic controls, which may be of benefit.[9, 10]

Furthermore, we tested if an extension of the duration of hypothermia to 60 min after reperfusion would have any beneficial effects. The result was compared to

a retrospective control group with a 15 minute duration of hypothermia after reperfusion. However, the effect of extended hypothermia was similar to the effect of short hypothermia. These findings could be important for the design of clinical hypothermia studies. The previous clinical trials have used 3 or 6 hours hypothermia after reperfusion. During this time the patient is sedated and immobilised. Since our animal data did not demonstrate an effect of prolonging the duration of treatment from 15 to 60 minutes, it appears logic to shorten the duration of hypothermia in a future clinical study; perhaps to 1 hour. This would simplify the protocol, be more comfortable for the patient and could possibly reduce complications.

Paper III was followed by a clinical proof-of-concept study, mainly designed to assess the safety and feasibility of combination hypothermia in STEMI-patients; the RAPID MI-ICE study.[108] We found that it was safe and feasible to induce hypothermia in awake patients with STEMI, without delaying reperfusion, and that hypothermia treatment reduces infarct size with 38%. This study confirmed the preclinical findings in a clinical material.

Cardioprotective mechanism of hypothermia

The exact mechanism through which therapeutic hypothermia exerts its tissue protective effect is not known, but it is thought to reduce the metabolic demand of the cells.[60, 61, 109, 110] However, reduced oxygen demand does not fully explain the positive effects of hypothermia and several additive effects have been suggested.[62] It has been shown that mild hypothermia can prevent ischemic cells from entering apoptosis through prevention of mitochondrial dysfunction and inhibition of caspase release.[111-113] Moreover, therapeutic hypothermia has been shown to improve ion homeostasis, suppress ischemia induced inflammatory reactions, decrease free radical formation, stabilize cellular membranes and prevent intracellular acidosis.[59] A large part of the knowledge about tissue protective effects of hypothermia stems from experimentation in cerebral ischemia/reperfusion models. Conceivably, hypothermia modulates similar processes in the heart.

The aim of paper IV was to evaluate the effect of mild hypothermia on ischemia related coronary t-PA release. The main finding was that mild hypothermia markedly reduces coronary t-PA release during the reperfusion phase, with mean peak value of total t-PA release 26 fold higher in the control group than in the hypothermia group.

The most prominent source of t-PA is the endothelial cell and the release of t-PA is triggered by various injurious stimuli including ischemia. The release is mediated by a variety of substances including thrombin, ATP, ADP, serotonin, bradykinin, and epinephrine.[39, 114-122] The effects of hypothermia are probably mediated either directly on the endothelial cells or by attenuating the accumulation of stimulators of the endothelium. We have previously shown that hypothermia reduces reactive hyperemia by 43%.[46] Interestingly, hypothermia abolished t-PA release above basal levels completely, indicating a more selective effect on the endothelial t-PA release. This discrepancy also rules out blood flow mediated shear stress as the only factor for t-PA release.

Under physiologic circumstances, t-PA is important in keeping vessel walls free of thrombi and during pathologic thromboembolic disease states t-PA is a vital component of the endogenous thrombolytic system. Thrombolytic treatment also utilize t-PA as the active component and such therapy has been shown to increase survival and functional outcome in stroke and myocardial infarct patients.[123, 124] However, t-PA appear to be a double-edged sword in the respect that it also has been found to have proinflammatory properties that could contribute to reperfusion injury.[41, 125, 126] t-PA is known to induce matrix degradation via activation of matrix metallopeptidase 9, and increase oxidative stress and inflammation via upregulation of inducible nitric oxide synthase.[127, 128] Furthermore, it is associated with activation and degranulation of mast cells with subsequent proinflammatory effects.[40] t-PA has also been shown to increase the release of norepinephrine from sympathetic neurons and thereby contribute to cardiac arrythmias in ischemia/reperfusion.[42] Norepinephrine also shifts the metabolic balance in an unfavourable direction by increasing oxygen demand via increased heart rate and inotropy, and decreasing oxygen availability by constriction of coronary arteries, and may thus aggravate the primary ischemia.[43, 44] Consequently, decreased norepinephrine release per se would also be expected to protect the ischemic myocardium. Taken together, it is possible that the hypothermia related reduction in t-PA release during ischemia/reperfusion contributes to the tissue protective effects of hypothermia. It may also be part of the explanation to why hypothermia has been found to increase defibrillation success and resuscitation outcome at cardiac arrest in a porcine model.[129]

Circulatory effects of hypothermia and impact on systemic t-PA release

The main findings of paper V were that cardiogenic shock resulted in increased basal levels of venous and arterial t-PA, as well as in net- and total t-PA release. Hypothermia inhibited any increase in t-PA. t-PA levels were found to correlate with both metabolic and hemodynamic parameters. Cardiogenic shock induced an inflammatory response with increased levels of TGF-β1 and IL-6, but hypothermia did not affect this response. As reported earlier, hypothermia was found to favourably affect hemodynamic- and metabolic variables.[69] In order to avoid possible bias from a reduction in infarct size, hypothermia was induced after the onset of reperfusion in this study.

Previous experimental studies have demonstrated that mild hypothermia increases myocardial contractility in excised heart preparations as well as in the in situ heart.[63-65] The increase in contractility is considered to be mediated by an increased myofilament sensitivity to existing Ca^{2+},[65] without a corresponding increase in myocardial oxygen consumption.[63, 64] A decrease in heart rate, combined with a larger stroke volume and an increased cardiac output has also been reported previously.[65] This is in line with the observations in the current experiment. Hypothermia has also been demonstrated to reduce the metabolic demand of the cells.[60, 61, 109, 110] In the current experiment, hypothermia treatment resulted in a significantly higher mixed venous saturation, pH and base excess. An explanation to the observed results could be that hypothermia lowered the peripheral oxygen demand, resulting in less tissue hypoxia and no development of metabolic acidosis.

Reduced oxygen demand does not fully explain the positive effects of hypothermia (please see previous section). In the current experiment hypothermia was found to inhibit a shock-related increase in basal arterial and venous levels of t-PA and also the release of t-PA from the peripheral vascular bed. It is possible that the release of t-PA was triggered by the shock related metabolic compromise. This hypothesis is supported by the observed inverse correlations between pH, base excess, mixed venous saturation and t-PA. However, even though the hypothermic animals were less metabolically compromised, they were still in shock and not metabolically unaffected. As hypothermia completely abolished t-PA release above basal levels, a more selective effect on the endothelial t-PA release is indicated. This hypothesis is further supported by the fact that significant correlations between t-PA and metabolic parameters were confined to the normothermic group.

In this experiment, cardiogenic shock was found to increase the anti-inflammatory TGF-β1 and pro-inflammatory IL-6, to a similar degree in both groups. The effect of shock on the pro-inflammatory TNF-∝ and anti-inflammatory IL-10 was neutral in both groups. Thus, a possible positive effect of t-PA on hemodynamic parameters does not involve these cytokines. However, t-PA has been reported to activate inducible nitric oxide synthase (iNOS),[128] which acts to increase the levels of nitric oxide (NO). In turn, NO has been associated with a reduction in myocardial contractility by uncoupling of calcium metabolism,[130, 131] through effects on glucose metabolism,[131] and through a reduction in β-adrenergic responsiveness.[132] Furthermore, NO induce vasodilatation, thereby decreasing coronary perfusion pressure and systemic perfusion.[11] Mild hypothermia has been demonstrated to reduce iNOS.[133, 134] In the current experiment, there was an inverse correlation between t-PA and systemic vascular resistance, mean arterial pressure and stroke volume. Possibly, the reduction in t-PA is a contributing link between hypothermia and improved hemodynamic status, via reduced iNOS levels.

CONCLUSIONS AND FUTURE PERSPECTIVES

- Inhibition of the activated complement factor C5a reduces myocardial ischemia/reperfusion injury and represents a novel treatment strategy with potential clinical applicability.
- Treatment with apyrase, according to a clinically applicable protocol, does not reduce myocardial ischemia/reperfusion injury.
- Therapeutic hypothermia reduces myocardial ischemia/reperfusion injury. It is vital that target temperature is reached prior to reperfusion. The combination of cold intravenous saline and endovascular cooling is fast and efficient. A short duration of treatment after reperfusion seems to confer similar benefit compared to a long duration.
- Mild hypothermia markedly reduces ischemia related coronary t-PA release. The reduction of t-PA release may contribute to the cardioprotective effect of hypothermia.
- Mild hypothermia improves hemodynamic and metabolic parameters in cardiogenic shock. This is associated with a reduction in basal t-PA levels and t-PA release from the peripheral vascular bed, but not with an altered inflammatory response as measured by IL-6, IL-10, TNF-α and TGF-β1.

Where do we go from here? Hypothermia treatment for cardioprotection should be taken one step further, to a phase II clinical trial. Such a trial is being planned, the CHILL-MI study. Hypothermia treatment for protection of the failing circulation should be evaluated in a phase I study. ADC-1004 is planned for toxicology testing and a dose-response experiment. After this, a phase I trial is planned. The use of ATP degrading enzymes did not have a cardioprotective effect in our large animal model. Clinical development is therefore not recommended.

Do these treatment strategies have the potential to change clinical practice? Yes, they have. However, despite decades of research there has been no successful translation of a cardioprotective treatment strategy into clinical practice, so a humble attitude appears appropriate.

Numerous cardioprotective strategies have consistently shown positive effects in animal models, and it is clear that reperfusion injury can be inhibited in these models. An important issue is the differences between these models and the clinical setting in which the therapy is thought to be applied. Experimental animals are typically young, healthy, lack concomitant medication and ischemia is brought about by the inflation of a balloon in an artery that is free from thrombus. Reperfusion is generally characterized by a very sudden, immediately maximized flow of blood. Patients, on the contrary, are often old, have co-morbidities and concomitant medication. They also have coronary heart disease and have often developed collateral vessels. Several animal experiments have shown difficulties in reproducing cardioprotective strategies under these circumstances.[135-138] Furthermore, ischemia in patients is often preceded by unstable angina with episodes of ischemia, hypothetically with a preconditioning effect. Reperfusion is often gradual with the initial passing of a guide-wire followed by balloon inflation a little later. Repeated occlusions during stent implantation may have a postconditioning effect. Moreover, a large minority of the patients reperfuse to some extent prior to percutaneous intervention, and cardiac reperfusion injury is well known to be impossible to attenuate once reperfusion is initiated. Taken together, this may blunt the efficacy of a novel treatment.[139-142]

One way forward could be the development of animal models that are more representative of the clinical situation. Moreover, in order to prevent inappropriate clinical trials, novel treatment strategies should be tested in several animal models prior to initiation of clinical experiments.

As ischemia/reperfusion injury is known to be multifactorial, the most promising approach appears to be a treatment with several effects on the pathologic process. Alternatively, a combination of several drugs aimed at different pathogenic mechanisms could be considered.

Once clinical studies are executed, it appears vital to primarily include previously healthy patients with a short duration of ischemia and a large ischemic territory. From the development of ischemia-reperfusion injury (Figure 1) it can be hypothesized that a short duration of ischemia would result in a comparatively large proportion of reperfusion injury. Furthermore, with a large area at risk, the absolute reduction in infarct size can be expected to be large as well. Sigma ST (summation of ST-segment elevation on electrocardiogram) could be used for screening. Patients with spontaneous reperfusion would have to be excluded. Importantly, effect on infarct size should be evaluated with MRI and adjusted for area at risk, also possible to assess with MRI.

All in all, one of the more promising concepts at the moment seems to be hypothermia treatment. It has a multifactorial mode of effect and is void of costly

development. With the large population of patients that would be eligible to cardioprotective treatment, and the potential benefits for these patients, further research is warranted.

SVENSK SAMMANFATTNING (Swedish summary)

Hjärtinfarkt beror på att flödet av blod i ett av hjärtats kranskärl har upphävts, oftast beroende på att ett åderförkalkningsplack brustit och att blod levrat sig i kärlet. Detta leder till att hjärtmuskel nedströms om stoppet dör av syrebrist och överskott på metabola slaggprodukter. Behandlingen syftar till att återupprätta flödet av blod, oftast genom ballongvidgning. Detta kallas reperfusionsbehandling.

I samband med reperfusionsbehandling avbryts vävnadsdöden nedströms om stoppet. Dock orsakar även reperfusion viss vävnadsskada, så kallad reperfusionsskada. Reperfusionsskadan uppstår till följd av flera olika mekanismer; bland annat inflammation, bildning av giftiga syreföreningar och skador på cellstrukturer till följd av obalans i salter. Det system av proteiner som annars används för att skydda kroppen mot bakterier, komplementsystemet, aktiveras också och skadar hjärtat. Samtidigt aktiveras system för att motverka dessa skador. Ett sådant system ändrar balansen mellan olika fosfatföreningar med minskad (skadlig) inflammation som följd.

Nettoeffekten av reperfusionsbehandling är alltså positiv, men det finns en negativ komponent (reperfusionsskada) som reducerar effekten. Genom att begränsa reperfusionsskadan så skulle den sammanlagda skadan på hjärtat i samband med hjärtinfarkt kunna minskas. I denna avhandling har reperfusionsskada och tänkbara behandlingsprinciper studerats. Arbetet har genomförts med hjälp av djurförsök i en grismodell.

I **arbete I** visas att hämning av komplementsystemet med hjälp av en substans vid namn ADC-1004 minskar reperfusionsskada, och skulle kunna användas för behandling av detta.

I **arbete II** visas att acceleration av det fosfatrelaterade vävnadsskyddande systemet inte bidrar till någon reduktion av skadan på hjärtat i samband med hjärtinfarkt.

I **arbete III** studeras kylbehandling. Kylbehandling innebär att kroppstemeraturen sänks till 33 grader med hjälp av kall dropplösning och en kylslang som placeras i den nedre hålvenen. Det visas att kylbehandling minskar skadan på hjärtat.

Kylbehandling verkar på flera av mekanismerna som bidrar till reperfusionsskada. I **arbete IV** studeras effekten av kylbehandling på en molekyl som heter t-PA. t-PA bidrar till den vävnadsskadande processen bland annat genom att öka inflammation. Vi visar att hypotermibehandling minskar frisättningen av t-PA från hjärtat i samband med hjärtinfarkt.

I samband med en stor hjärtinfarkt kan hjärtat skadas så pass mycket att hela blodcirkulationen sviktar. Sådan svikt förvärras av en inflammatorisk reaktion i hela blodcirkulationen. Tillståndet kallas kardiogen chock. I **arbete V** visas att kylbehanding förbättrar blodcirkulationen i samband med kardiogen chock och att detta korrelerar med en minskad frisättning av t-PA från blodkärlen ute i kroppen.

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