Pharmacological interventions against myocardial ischaemia and reperfusion injury

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ABSTRACT

Background: Although the concept of early restoration of coronary blood-flow constitutes an important factor to reduce the injury caused by myocardial ischaemia, reperfusion in itself can aggravate the damage to myocardial tissue, a phenomenon denoted myocardial reperfusion injury.

Aims: To investigate whether two different pharmacological interventions, cyclosporine A (CsA) and the novel enkephalin analogue EP 94, could confer cardioprotection in porcine models of myocardial ischaemia and reperfusion. Furthermore, to examine the distribution of the opioid receptor subtypes in the porcine heart, and to investigate how this expression is affected by ischaemia and reperfusion.

Methods: Anesthetised pigs underwent balloon occlusion of the left anterior descending coronary artery, followed by reperfusion. CsA and EP 94 were administered at reperfusion and hearts stained to measure infarct size. mRNA and protein expression of pro-apoptotic proteins, endothelial NO-synthetase and opioid receptor subtypes was quantified in the control and ischaemic/reperfused areas.

Results: Two different dosages of CsA did not confer cardioprotection whereas EP 94 reduced myocardial infarct size in a dose-dependant manner. Protein expression of the κ- and δ-opioid receptors was detected in the left ventricle, with an up-regulation of the δ subtype after ischemia and reperfusion. The μ-opioid receptor was not detected.

Conclusions: CsA did not reduce myocardial infarct size, whereas the novel enkephalin analogue EP 94 conferred cardioprotection in different porcine models. The κ- and δ-opioid receptors were detected in the pig left ventricle.

Keywords: myocardial ischemia, reperfusion injury, opioids, cyclosporine A

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LIST OF PAPERS

This thesis is based on the following studies:

I

Karlsson LO, Zhou AX, Larsson E, Åström-Olsson K, Akyürek LM, Grip L.

Cyclosporine does not reduce myocardial infarct size in a porcine ischemia-reperfusion model.

J Cardiovasc Pharmacol Ther 2010 Jun; 15(2): 182-9

II

Karlsson LO, Bergh N, Grip L

Cyclosporine A, 2,5 mg/kg, does not reduce myocardial infarct size in a porcine model of ischemia and reperfusion

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III

Karlsson LO, Grip L, Bissessar E, Bobrova I, Gustafsson T, Kavianpour M, Odenstedt J, Wikström G, Gonon AT

Opioid receptor agonist Eribis peptide 94 reduces infarct size in different porcine models for myocardial ischemia and reperfusion

Eur J Pharmacol. 2011 Jan 25;651(1-3): 146-51

IV

Karlsson LO, Bergh N, Li L, Bissessar E, Bobrova I, Gross GJ, Akyürek LM, Grip L

Dose-dependent cardioprotection of enkephalin analogue Eribis peptide 94 and cardiac expression of opioid receptors in a porcine model of ischaemia and reperfusion

Submitted

TABLE OF CONTENTS

ABBREVIATIONS

AAR	area at risk
AIF	apoptosis inducing factor
ANT	adenine nucleotide translocase
ATP	adenosine triphosphate
CsA	cyclosporine A
CypD	cyclophilin D
ECG	electrocardiogram
EP 94	eribis peptide 94
IM	inner membrane
IPC	ischaemic preconditioning
IPost	ischaemic postconditioning
IS	infarct size
LAD	left anterior descending artery
MPTP	mitochondrial permeability transition pore
NO	nitric oxide
NOS	nitric oxide synthethase
OM	outer membrane
PCI	percutaenous coronary intervention
RISK	reperfusion injury salvage kinases
ROS	reactive oxygen species
RT-PCR	real time polymerase chain reaction
TTC	triphenyltertrazolium-chloride

TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
SEM	standard error of mean
SD	standard deviation

INTRODUCTION

Ischaemic heart disease is the leading cause of death in the industrialized world and an increasing problem in developing countries. The different presentations include sudden death, acute myocardial infarction, stable angina pectoris, arrhythmias and congestive heart failure. Although progress has been achieved over the last few decades for the treatment of acute myocardial infarction with the introduction of pharmacological and mechanical intervention reperfusion strategies, acute myocardial infarction remains a major cause of death and disability, making the development of further adjunctive therapies a desirable task.

In the 1970s it was concluded that early reperfusion of an occluded coronary artery could salvage myocardial tissue[1-2]. This knowledge was translated to the clinical setting with the introduction of thrombolytic therapy[3-4] and, subsequently, percutaneous coronary intervention (PCI)[5]. Limitation of infarct size has since evolved as an important factor in reducing the consequences of acute myocardial infarction[6-8]. However, reperfusion *per se* can mediate negative effects, and restoration of blood flow can aggravate the damage to the myocardial tissue, a phenomenon first described by Jennings in the 1960s[9]. This concept, defined as reperfusion-induced cell injury to the cardiomyocytes still viable at the end of ischaemia, is denoted myocardial reperfusion injury[10]. Since the initial description, this harm has evolved as an important contributor to cardiomyocyte injury caused by acute myocardial infarction and subsequent reperfusion, with experimental models indicating this detriment to account for approximately 50% of the final infarct size[10].

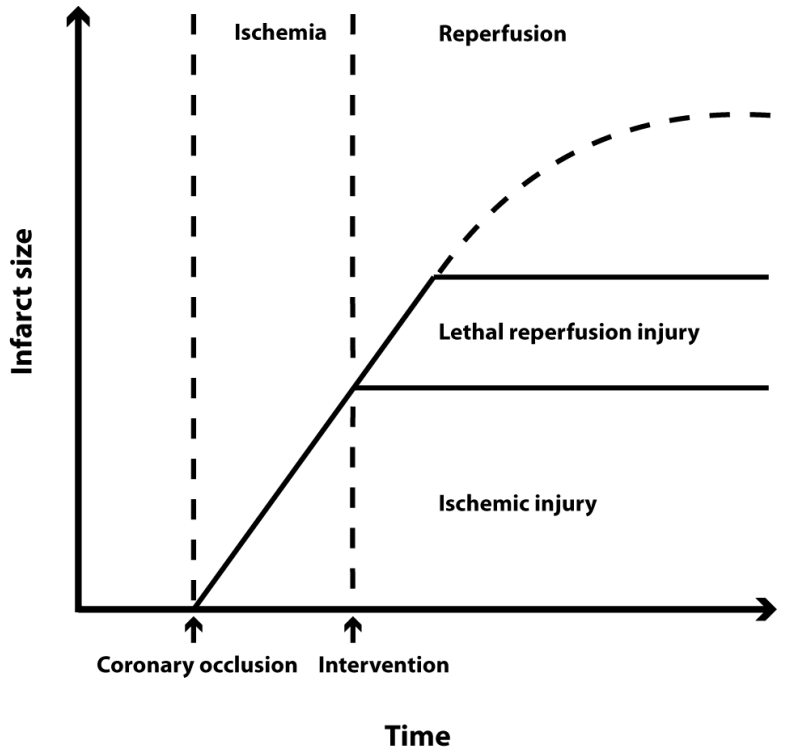

Figure 1. *Lethal reperfusion injury. Modified after Garcia-Dorado. Cardiovascular research. (2006) 69 (1): 1-3*

Classically, the injury induced to the myocardium during reperfusion is divided into four different entities: myocardial stunning, reperfusion arrhythmias, no-reflow phenomenon and lethal reperfusion injury[10]. Whereas these are all potentially harmful in the clinical setting, the irreversible nature of lethal reperfusion injury makes it the most interesting target in order to salvage the reperfused myocardium.

Cellular effects of ischaemia

Following acute occlusion of a coronary artery, presumably through a thrombus formation, oxygen supply to the jeopardized myocardium drops. This leads to a switch in metabolism from aerobic oxidative phosphorylation in the mitochondria to anaerobic glycolysis in the cytosol, with subsequent accumulation of protons (H^+) and lactic acid. The tissue acidosis is followed by a gradual cessation of adenosine triphosphate (ATP) production. In order to attempt to correct the intracellular acidic milieu, cardiomyocytes activate the Na^+/H^+-antiporter in order to pump H^+ out of the cell, resulting in a high intracellular concentration of Na^+. Because of the lack of ATP, the Na^+/K^+ ATPase works inefficiently and Na^+ cannot be extruded. This activates the Na^+/Ca^{2+} exchanger in reverse mode, leading to a high intracellular concentration of Ca^{2+}. Altogether, a failure of maintaining ionic homeostasis follows, resulting in cellular contracture[11-12]. Additionally, if no restoration of circulation is accomplished, the jeopardized myocardium will undergo necrosis, commencing in the endocardium after approximately 30 minutes and propagating towards the epicardium[13-14]; the "wavefront phenomenon"[15]. If, on the other hand, coronary blood flow is re-established by means of either lytic therapy, PCI or acute coronary artery bypass grafting, the oxygen makes it possible for the mitochondria to respire once again, mediating the return of ATP synthesis through oxidative phosphorylation, with possible survival of the cell.

Reversible and irreversible reperfusion injury

As mentioned above, the return of blood flow to the myocardium can also mediate reperfusion injury, classically divided into two different types: reversible and irreversible forms. The no-reflow phenomenon, defined as obstructed microvascular blood flow despite an open infarct-related artery is often characterized as both reversible and

irreversible[16]. Myocardial stunning (characterized by a delayed recovery of myocardial function despite restoration of coronary blood flow) and reperfusion arrhythmias, on the other hand, both constitute different forms of reversible injury[10, 17], indicating that the return of blood flow can eventually resolve this harm. Reperfusion, however, is also followed by a pronounced production of reactive oxygen species (ROS), high intracellular Ca^{2+} concentration and restoration of normal pH. Furthermore, release of cell adhesion molecules during reperfusion mediates neutrophil aggregation. In concert, these factors are known to cause the irreversible injury known as *lethal reperfusion injury*, with the mitochondria as a possible end-effector.
Following is an overview of the different mechanisms responsible for lethal reperfusion injury and a presentation of the therapeutic interventions investigated in order to reduce this harm.

The mitochondrial permeability transition pore (MPTP)

The mitochondria are the organelles responsible for energy supply in the cell with production of ATP through oxidative phosphorylation. They are surrounded by a membrane bilayer; the inner (IM) and outer membranes (OM), which are composed of phospholipids with different integrated proteins. The IM is folded and highly impermeable to all but a few selected metabolites and ions, whereas the OM is more permeable except for larger molecules, but it is also more rigid. Between the two membranes is the intermembrane space, which has the same ionic composition as the cytosol because of the permeability of the OM[18].
Also located in the membranes is the mitochondrial permeability transition pore (MPTP), assumed to be an important participant in mitochondria function and pathology. Despite enormous attention over the last few decades, however, the definite molecular structure of the MPTP is not known[19], even though recent research implicates that it consists of at least one component in the IM, adenine nucleotide translocase (ANT), and a regulatory component in the mitochondrial

cytosol, cyclophilin D (CypD). These are both thought to regulate the core component of the MPTP that spans the two membranes[19].

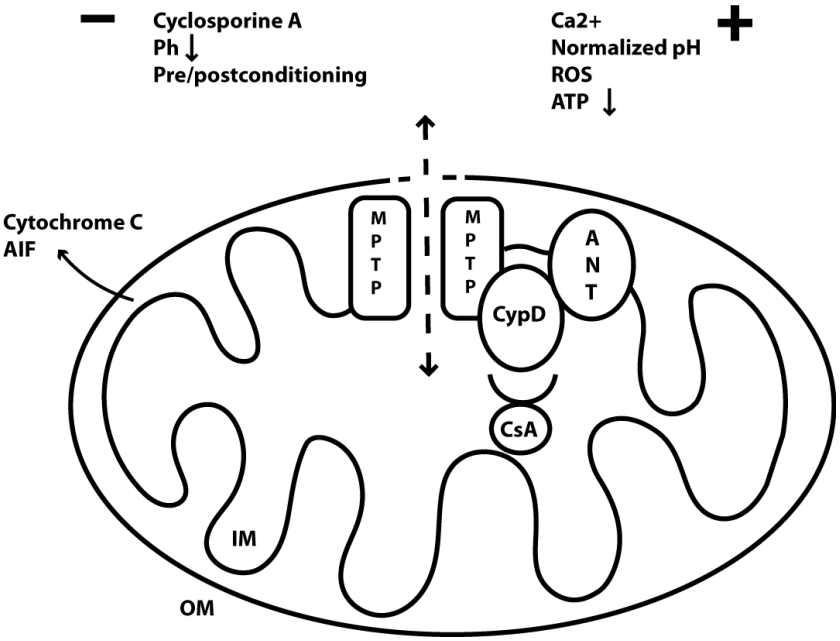

Figure 2. *Mitochondrial permeability transition pore. See text for details.*

In the physiological setting MPTP remains closed, only allowing diffusion of certain molecules over the membrane bilayer. Under certain conditions, however, the MPTP opens and becomes permeable to molecules with a molecular weight < 1.5 kDa. If it remains open, this will lead to the uncoupling of oxidative phosphorylation, collapse of the electrical gradient (mitochondrial transition) and, finally, the activation of proteases, nucleases and phosphatases, leading to cell necrosis. On the other hand, if the MPTP closes, the transient permeability of the membranes leads to an osmotic swelling of the mitochondria. This is well tolerated by the heavily folded IM, but may lead to rupture of the more rigid OM with a subsequent leakage of proapoptotic proteins located in the intermembrane space.[20].

The factors known to mediate MPTP opening include ATP depletion, oxidative stress, Ca^{2+} overload and a high concentration of inorganic phosphate, i.e. factors that are all abundant during ischaemia. The last remaining component, the rapid restoration of physiological pH, is fulfilled by the return of blood flow, and all restraints on the permeability of the mitochondrial membranes are thus removed[20]. This would indicate that the pore remains closed during ischaemia and opens during reperfusion, a fact supported by numerous data[18]. In line with this knowledge the MPTP has been suggested to be a core component of lethal reperfusion injury[21-23]. Furthermore, several cardioprotective interventions have been shown to inhibit MPTP opening. These include pre- and postconditioning (see below)[24-25], the antioxidant compounds pyruvate[26] and propofol[27], volatile anaesthetics[28] and cyclosporine A (CsA). Indeed, CsA has, since the original description by Crompton et al in 1988, served as the model compound for the inhibition of MPTP opening[29], possibly through binding of the regulatory component CypD. Since the initial reports, numerous experimental studies in different models have shown a cardioprotective effect with the administration of CsA at reperfusion, even though the results are disparate[22, 30-33].

Reactive oxygen species

ROS are molecules containing oxygen with one or more unpaired electrons, making them highly reactive and unstable. Reperfusion of the ischaemic myocardium leads to an abrupt increase in the production of ROS, and several studies implicate a pivotal role for these molecules in lethal reperfusion injury[34-37]. This is sometimes referred to as the “oxygen paradox”, highlighting the fact that the return of oxygen aggravates the injury caused by ischaemia alone[38]. The principle sources for the generation of ROS at reperfusion are the re-energized transport chain in the mitochondria[10, 39], neutrophils[40], and xanthine oxidase, with the substrates xanthine and hypoxanthine accumulating during ischaemia[41-42]. The injury induced by ROS

includes the consumption of nitric oxide (NO), making it impossible for this compound to exert its cardioprotective effects[43]. Furthermore, ROS has a direct damaging effect on proteins, nucleic acids and lipids[44]. Finally, as described previously, ROS is thought to mediate MPTP opening, contributing to a feedback loop of additional ROS being released from the mitochondria[45].

As in the case with MPTP, numerous interventions have been tested in order to reduce reperfusion injury by inhibiting ROS formation including: superoxide dismutase[46-47], vitamin E[48], the free radical scavenger edaravone[49-50], xanthine oxidase inhibitor allopurinol[51] and the anti-anginal drug trimetazidine[52]. Although promising results have been presented in the preclinical setting, experience in the clinic has been largely disappointing[52-55].

Inflammation

After restoration of coronary blood flow, complement activation, cytokines and release of ROS from endothelial cells and cardiomyocytes promote the infiltration of neutrophils into the myocardium[56-57]. Data from preclinical studies indicate that neutrophils accumulate in the intravascular space for the first 6 hours, whereafter they advance into the myocardium during the next 24 hours[58]. Once the cells have migrated into the parenchyma they can impair the tissue in a number of ways: release of degradative proteases and ROS, microembolization in small vessels, damage to vascular endothelium and release of further mediators that attract even more neutrophils[40].

Interventions in the experimental setting reduce infarct size when neutrophils and inflammatory pathways have been targeted. Specific filters[59] and neutrophil-depleted blood[60-61] have been used in order to reduce the amount of neutrophils in the reperfused tissue. Additionally, antibodies against molecules responsible for cell adhesion, including CD-11[62] and CD-18[63], P-selectin[64] and intracellular adhesion molecule-1[65] confer cardioprotection in different animal models. None

of these therapies, however, have proved beneficial in the clinical setting[10]. Indeed, the only intervention targeting the inflammatory pathway with a proven benefit in humans is adenosine, an autacoid interacting with G-protein-coupled receptors. Even though known to affect different cell types, the cardioprotective effect is most often attributed to an inhibition of neutrophil function[40]. In the Amistad-II trial, an 11% reduction of infarct size was noticed with the administration of adenosine to patients undergoing acute PCI, with no proven benefit in terms of clinical outcome[66]. However, in a post-hoc analysis, adenosine administered to patients with anterior infarction presenting within 3 hours enhanced early and late survival[67].

Calcium

Under basal conditions there is a 10 000-fold concentration gradient for Ca^{2+} across the plasma membrane with the highest concentration in the extracellular space, enabling a rapid increase in cytosolic Ca^{2+} in order to regulate fundamental cell functions. The negative potential on the inside of the plasma membrane further enhances this driving force for Ca^{2+} influx. The action potential in the cardiomyocyte is initiated by an influx of Na^{+}, but the depolarization is prolonged through the opening of L-type Ca^{2+} channels in the sarcolemma, leading to an influx of Ca^{2+} into the cytosol. This in turn activates the ryanodine receptor in the sarcoplasmic reticulum (SR), leading to the release of stored Ca^{2+} from this compartment. This "calcium-induced calcium release" leads to cross bridging between myosin and actin with muscular contraction as a consequence. Furthermore, Ca^{2+} binds to the regulatory protein calmodulin, mediating a broad regulatory action in the cell by the activation of different proteins, including serine/threonine-specific protein kinases, phosphatases and nitric oxide synthases [68].

As previously described, ATP depletion and ionic fluxes lead to high intracellular concentrations of Ca^{2+}, Na^{+} and H^{+} during ischaemia. At reperfusion, the return of ATP reactivates the Na^{+}/K^{+} ATPase in the

sarcolemma and the Ca^{2+} ATPase in the SR. If the capacity to accumulate Ca^{2+} in the SR is exceeded, oscillations in Ca^{2+} concentration will commence. The cell then relies on the possibility to extrude Ca^{2+} with the Na^{+}/Ca^{2+} exchanger working in "forward mode", which depends on the restoration of a transsarcolemmal gradient of Na^{+}, i.e. a properly working Na^{+}/K^{+} ATPase. On the other hand, if the intracellular Ca^{2+} concentration remains uncorrected, myofibrillar hypercontracture, cytoskeletal damage and MPTP opening will follow, leading to cell necrosis[11, 18].

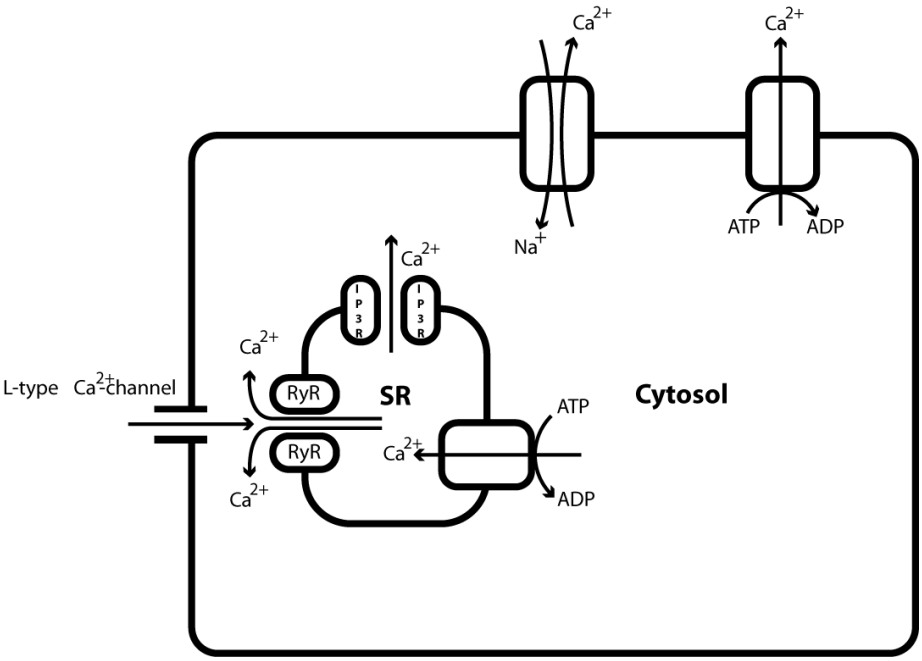

Figure 3. *Calcium handling in the cardiomyocyte.*

As in the case with other interventions targeting lethal reperfusion injury, attenuating Ca^{2+} overload at reperfusion has been shown to reduce infarct size by approximately 50% in different animal models[10]. Interventions include the inhibition of L-type channels with diltiazem and clevidipine, with conflicting results in porcine models[69-71], and no proven benefit in humans[72]. Administration of Na^{+}/H^{+} exchanger inhibitors mediates cardioprotection when administered before the

onset of ischaemia in experimental models[73-75], but intervention at reperfusion has not conferred cardioprotection in the clinical setting[72, 76-78].

Apoptosis in lethal reperfusion injury

Programmed cell death, apoptosis, is involved in various pathologies, including ischaemic heart disease[79]. The literature is, however, conflicting, in part owing to the fact that different methods have been used in order to detect apoptosis[79-80]. The histological morphology characteristics include chromatin condensation, DNA fragmentation, bulging of the plasma membrane (blebbing) and cell shrinkage[81]. The process implies phagocytosis by adjacent cells without an inflammatory reaction, a process dependent on ATP consumption[68].

There are two different pathways for apoptotic induction: extrinsic (death receptor pathway) and intrinsic (mitochondrial pathway) signaling. The former include NO, toxins, Fas ligand, and different cytokines, including tumour necrosis factor-alpha. After binding to different receptors, these activate a cascade of caspases, carrying out the programmed cell death. The intrinsic pathway can be activated by, among other mediators, viral infections, radiation and, relevant in the setting of lethal reperfusion injury, Ca^{2+} overload and hypoxia[82]. The main target for these intracellular signals is the mitochondria. As described previously, the intracellular milieu at the time for reperfusion, with Ca^{2+} overload, ATP depletion, oxidative stress and eventually restoration of physiological pH mediates opening of the MPTP[20]. Furthermore, although not fully elucidated, the permeability of the outer mitochondrial membrane seems to be regulated by a family of proteins called the Bcl-2 group, possibly through interaction with the MPTP [83]. The subsequent swelling of the mitochondria and, eventually, rupture of the OM leads to leakage of different proteins located in the intermembrane space, among these cytochrome C and apoptosis inducing factor (AIF)[18]. Cytochrome C initiates the caspase

cascade with activated caspase-3 as the end-effector[84-85] whereas AIF translocates to the nucleus, causing chromatin condensation and DNA fragmentation independent of caspase activation[86].

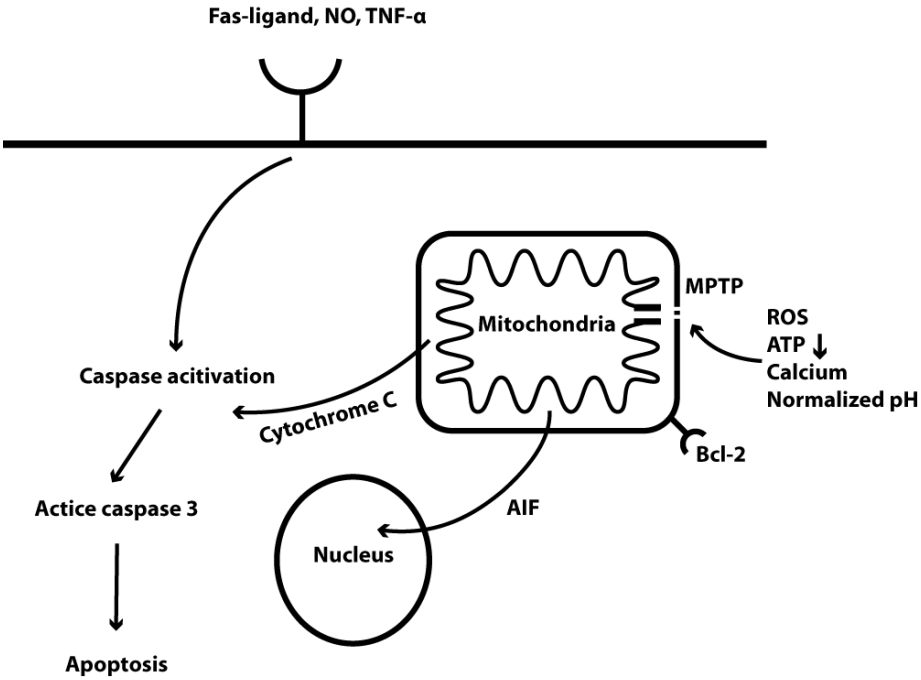

Figure 4. *Apoptosis in myocardial reperfusion injury. See text for details.*

There is a general agreement that apoptosis contributes to the final infarct size in acute myocardial infarction, but the extent of this injury is presently unclear. Furthermore, to what extent reperfusion *per se* or ischaemia alone mediates this harm remains a matter of debate[79-80]. This accounts for the fact that despite promising results from animal studies, the clinical implication for intervention against the apoptotic pathways remains elusive[79-80].

Nitric oxide

NO is an important messenger molecule with the ability to control vascular tone[87]. The principle source of NO formation is catalyzation of the precursors L-arginine, oxygen and NADPH by the enzyme nitric oxide synthase (NOS). There are three different isoforms of the enzyme: neuronal (nNOS) and endothelial (eNOS) which are both constitutively expressed, and the inducible isoform (iNOS)[88]. Whereas high concentrations of NO react with superoxide to form the highly reactive and potentially harmful oxidant peroxynitrite, low levels confer protection in the heart after a period of ischaemia and reperfusion[89]. The cardioprotective properties of NO are attributed to inhibition of neutrophil adherence to vascular endothelium, vasodilation, prevention of platelet aggregation, and reduced apoptosis through inhibition of caspase-3 activation[90-93]. The importance of NOS in this setting has also been investigated: overexpression of eNOS mediates cardioprotection, whereas deficiency of the enzyme aggravates reperfusion injury[94-95]. Furthermore, the different isoforms of the enzyme have been shown to play pivotal roles in pre- and postconditioning, as well as mediating the beneficial effect of pharmacological treatment with ACE- inhibitors, statins and phosphodiesterase-5 inhibitors in the experimental setting[96-98].

Pre- and postconditioning

Although not investigated in the present thesis, pre- and postconditioning will be briefly reviewed due to their importance in the understanding of myocardial ischaemia and reperfusion injury. In 1986, Murry et al first described that repetitive brief occlusions of a coronary artery prior to infarct induction could limit infarct size, a phenomena denoted ischaemic preconditioning (IPC)[99]. This protective effect has since been repeated in numerous species, including humans[100]. The protection consists of two different phases, an early

phase lasting for a few hours after the index ischaemia, and a second phase that appears after 12-24 hours and lasts for 2-3 days[101]. IPC was later extended to the concept of remote IPC, where repetitive periods of ischaemia to organs distant to the heart conferred protection of the myocardium during ischaemia and reperfusion[102]. Both interventions, however, require the preconditioning protocol to be applied before the onset of the ischaemic insult, a fact that made the concept difficult to apply in the clinical setting. In 2003, Zhao et al reported that brief episodes of ischaemia applied at the onset of reperfusion, ischaemic postconditioning (IPost), reduced infarct size in dogs[103]. With the advantage of application during reperfusion, the concept was translated to the clinical setting where Staat and co-workers could report a reduction of infarct size with IPost, with an improved left ventricular ejection fraction after 1 year[104-105]. These findings have later been repeated in several small clinical trials[106], even though the results are somewhat conflicting[107].

The triggers of protection that IPC and IPost are thought to recruit include, among others, adenosine, bradykinin and opioids. These in turn are thought to activate a cascade of prosurvival kinases known as the reperfusion injury salvage kinase (RISK) pathway, with subsequent closure of the mPTP and opening of mK(ATP) channels[80].

Opioid peptides in myocardial ischaemia and reperfusion

The analgesic effect of morphine via actions on the central nervous system is well known. However, the fact that myocardial tissue contains opioid receptors and is able to synthesize opioid peptides has only recently been discovered, and it is now appreciated that opioids regulate the heart during both normal conditions and disease states[108]. The three known opioid receptors subtypes, μ, κ, and δ, are G-protein coupled receptors and associated with endorphins, dynorphins and

enkephalins, respectively. Whereas all different opioid peptides are synthesized in the myocardium[108], the expression of the different opioid receptor subtypes remains conflicting, with possible differences between species[109-110]. In the rat heart, the μ-opiod receptor vanishes during adolescence[111], whereas δ- and κ-opioid receptors are expressed in adult cardiomyocytes[112-114]. This is quite different from the human heart, where μ- and δ-opioid receptors are expressed in both ventricles, whereas the κ-opioid receptor seems to be absent, or only vaguely expressed[110, 115-116]. Information regarding receptor expression in other species is, to a large extent, lacking[109]. Furthermore, how the receptor expression is affected by ischaemia and reperfusion remains largely unknown.

Apart from their analgesic effect, opioid peptides have been shown to confer cardioprotection when administered in the setting of myocardial ischaemia and reperfusion[117]. This effect is most often attributed to stimulation of the δ-opioid receptor, but all three receptor subtypes have been implicated as responsible targets[117-120]. The protective effect "downstream" from receptor activation is attributed to activation of different prosurvival kinases, including phosphatidylinositol 3-kinase pathway (PI3K), protein kinase C-δ (PKC-δ), serin-threonin kinase Akt and extracellular signal-regulated kinase (ERK). Other pathways that have been implicated include opening of mitochondrial K(ATP) channels and the inhibition of both glycogen synthase kinase-3 β and MPTP opening[115, 121-126]. Furthermore, cardioprotection by other compounds targeting G-protein coupled receptors (e.g. adenosine and bradykinin) is mediated through an up-regulation of endothelial NO synthase (eNOS)[127-128]. This has not, however, been investigated in the setting of opioid administration at reperfusion.

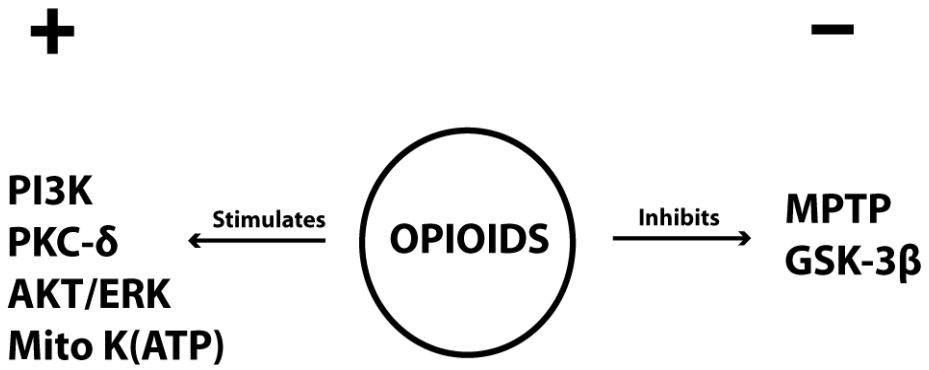

Figure 5. *Proposed mechanisms for the cardioprotective effects of opioids. See text for details.*

Eribis Peptide 94 (EP 94) is a novel enkephalin analogue thought to interact with the μ- and δ- opioid receptors and, to a lesser extent, with the κ-opioid receptor. In preliminary rat experiments, EP 94 confers protection against myocardial reperfusion injury when administered both during ischaemia and at reperfusion.

Rationale for the porcine model

As outlined above, impressive results regarding infarct size reduction have been obtained in animal models when applying different treatment strategies at reperfusion, with most regimens, unfortunately, failing when translated to humans[10, 129]. Possible explanations for this discrepancy are numerous. In the experimental setting, coronary ischaemia is induced in an otherwise healthy and young (often male) animal without concurrent medication. In contrast, when examined in the clinical setting, the intervention against lethal reperfusion injury is generally administered to an elderly person with concomitant diseases requiring medication. Another difference is the genesis of myocardial ischaemia; in animal models this is most often obtained through external ligation of a coronary artery, whereas in humans a coronary

occlusion is the consequence of a thrombus formation, a condition notoriously difficult to recreate in experimental models in a standardized way.

Other disparities include the administration regimen of the drug, with variation by means of the routes used and the duration of the administration. Last but not least, species differences obscure the picture; most results are obtained in mice and rat models, animals known to possess considerable differences when compared with humans. The pig, on the other hand, holds a great deal of homology with humans, having approximately the same heart size and a coronary artery anatomy that resembles humans, but without collateral development[130]. Furthermore, the size of the pig makes it feasible to perform a closed-chest protocol, with the possibility to induce coronary occlusion by balloon inflation. This allows greater resemblances to the clinical situation, without external trauma through ligation of the vessel, and eliminates the possible confounder of hypothermia that can be encountered in open-chest models[131-132].

Although dependent on the definition of a "positive result", one could argue that at least two known interventions against lethal reperfusion injury have demonstrated promising, but not unequivocal, results in the clinical setting, i.e. postconditioning[104] and adenosine[66]. These interventions have also been shown to confer cardioprotection in different porcine models[133-134], indicating this species as an attractive one before translating a possible intervention to humans. This reasoning led us to use our porcine models to examine two different interventions in order to try to reduce lethal reperfusion injury.

AIMS OF THE THESIS

- To investigate if cyclosporine A reduces infarct size and apoptosis in porcine models of myocardial ischaemia and reperfusion.
- To evaluate the cardioprotective effect of the novel enkephalin analogue EP 94 in different porcine models of ischaemia and reperfusion, and to see if a possible beneficial outcome could be mediated through NO-dependent mechanisms.
- To examine the distribution of the opioid receptor subtypes in the porcine heart, and to investigate how this expression is affected by ischaemia and reperfusion.

MATERIAL & METHODS

Ethics

All animal procedures conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication 85-23, revised in 1985). Approval for the different protocols was obtained from the local animal ethics committee.

General considerations

A total number of 138 pigs (27-49 kg) were used in the four different protocols described in the following. Throughout the experiments, if not stated otherwise, the pigs were randomized to their different treatment groups and all interventions and measurements were performed in a blinded manner to the investigator.

The porcine models - ischaemia and reperfusion procedure

Three different models of ischaemia and reperfusion were used throughout the thesis. The closed-chest model used in papers II-IV will be presented in detail, and the two different open-chest models (papers I and III, respectively) will be discussed briefly thereafter.

Closed-chest model, papers II-IV: The pigs were premedicated with acetylsalicylic acid and sedated with intramuscular tiletamine and zolazepam. After administration of atropine, general anaesthesia was induced by an intravenous injection of pentobarbital, and mechanical ventilation with 40% oxygen was performed via oral intubation. Anaesthesia and analgesia was thereafter maintained by continuous

intravenous infusions of pentobarbital and morphine, respectively. Venous access was established by a 5 French (Fr) introducer sheath through the right jugular internal vein and monitoring of mean arterial pressure and heart rate was achieved through an arterial cannula in the femoral artery. An 8 Fr introducer sheath was inserted into the right carotid artery enabling catheterization. Temperature was maintained at 37.5-39°C, if necessary through external heating.

At the commencement of catheterization all pigs received unfractionated heparin, which was repeated every hour. After stabilization, a 6 Fr PCI-guiding catheter was inserted via the arterial introducer sheath and placed in the left coronary ostium under X-ray guidance. An angioplasty balloon was inflated in the left anterior descending artery (LAD) distal to the second diagonal branch. Occlusion was verified angiographically and through ST-segment alteration on the ECG. After 40 minutes of occlusion the balloon was deflated and reperfusion verified by contrast injection. The pigs were then kept under anaesthesia for four hours, and thereafter sacrificed.

Open-chest model, paper I: In this protocol, an open-chest model was used, a concept that later developed to the closed-chest protocol used in papers II-IV. The differences compared with the closed-chest model include premedication with midazolam and ketamine, and fentanyl being infused for analgesia. Furthermore, an open-chest approach was used with a sternotomy performed from the beginning of the experiments, whereas the coronary occlusion was accomplished in the same manner, i.e. by inflation of an angioplasty balloon. The coronary occlusion was, however, maintained for 45 minutes and the reperfusion phase lasted for two hours. Finally, lidocaine was administered in order to reduce ventricular arrhythmias, and in a subset of the pigs isoflurane was used as a general anaesthetic.

Open-chest model, paper III: These experiments were carried out at the Karolinska University Hospital, Solna, Stockholm, Sweden. The pigs received the same premedication and drug administration regimen

throughout the experiment as in the closed-chest model, except for a lower dosage of morphine being infused. However, myocardial ischaemia was obtained through a ligature around the LAD, requiring the open-chest approach. Furthermore, the administration route for the investigated compound was through a thin catheter placed in the LAD distal to the ligature, admitting drug infusion into the jeopardized myocardium during ischaemia.

Analysis of CsA and EP 94 blood concentration

CsA and EP 94 blood concentrations were analyzed in pig plasma using a chemiluminescent microparticle immunoassay and electrospray ionization in positive mode, respectively.

Infarct size measurements

At the end of reperfusion, a sternotomy was performed (in the closed-chest model). The angioplasty balloon was inflated at the same location as during ischaemia induction. In the open-chest model (paper III), the ligature around the LAD was re-occluded. Evans blue was infused via the central venous catheter, followed by an injection of a lethal dose of potassium chloride. The heart was excised and the right ventricle and atria removed. The left ventricle was sliced in approximately 10 mm thick slices and incubated for 10 min at 37°C in 2, 3, 5-triphenyltetrazolium-chloride (TTC). The area at risk (AAR) and infarct size (IS) were defined as the areas not stained by Evans blue and TTC, respectively, and delineated by planimetry. Additionally, the mean weights of AAR and IS were calculated.

Histological processing

Harvesting of biopsies

Myocardial biopsies were taken from the control (free lateral wall) and ischaemic/reperfused areas (i.e. the lateral border of the area at risk, presumed not to be infarcted) and immediately frozen in liquid nitrogen and stored at -70°C until further analysis.

Real-time RT-PCR

Samples were homogenized and RNA was isolated using RNeasy Mini spin columns. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kits. RT-PCR was performed to detect messenger RNA (mRNA) expression with relevant primers and probes. Relative levels of mRNA were calculated using the standard curve method and normalized to 18s ribosomal RNA.

Immunoblotting

Proteins in total homogenized cellular extracts were separated by SDS-PAGE and blotted onto a polyvinyl difluoride membrane. In summary, blocking was performed in phosphate-buffered saline with 5% non-fat milk, followed by incubation with appropriate antibodies in buffered saline with 5% non-fat milk. Immunoreactive bands were visualized with horseradish peroxidase conjugated secondary antibodies. Bands were thereafter densitometrically analyzed and their readings carried out using ImageQuant software.

Immunohistochemistry

Frozen cardiac biopsies were sectioned and stained with antibodies against κ- and δ-opioid receptors. The avidin-biotin detection system

was used, and the reaction was visualized by the substrate 3,3'-diaminobenzidine. Sections were slightly counterstained with hematoxylin and mounted with cellulose triacetate.

Determination of apoptosis by TUNEL staining

Apoptotic nuclei were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining from the control, ischaemic/reperfused and infarct areas. Briefly, the sections were incubated with terminal deoxynucleotidyl transferase and fluorescein-labeled dUTP. To identify all the nuclei, the sections were stained with blue fluorescent DAPI nucleic acid stain. To obtain the percentage of apoptotic cells, the number of TUNEL-positive cells was divided by the number of total DAPI-stained cells.

Intervention protocols and further analysis

In the present thesis, two different concepts in order to reduce lethal reperfusion injury were investigated: two dosages of CsA (papers I and II), and a conceptual test of the administration of enkephalin analogue EP 94 (paper III) followed by a dose-finding study for the same compound (paper IV).

Paper I

The study examined the cardioprotective effect of the administration of 10 mg/kg CsA at reperfusion in an open-chest model. In the first phase, 20 pigs were randomized to receive an intravenous infusion of either 10 mg/kg CsA (Sandimmune, Novartis, Basel, Switzerland) or vehicle for 3 minutes before reperfusion. Due to conflicting results in this phase, the study was expanded with a second phase where 16 pigs

anesthetized with pentobarbital were randomized to receive CsA or vehicle as described above.

Further analysis: RT-PCR and immunoblots were performed in order to detect proapoptotic proteins AIF, activated caspase-3 and anti-BCL2/adenovirus E1B 19 kd-interacting protein 3 (Bnip-3) in the left ventricle, and a TUNEL staining was carried out. The blood concentration of CsA was measured 45 minutes after administration.

Paper II

Despite the negative results obtained in paper I, a small study in humans reporting beneficial effects after administration of 2.5 mg/kg CsA in acute myocardial infarction[135] led us to investigate this administration regimen in our closed-chest model. A total of 24 pigs were randomized to receive either 2.5 mg/kg CsA (Sandimmune, Novartis, Basel, Switzerland) or saline given as a bolus injection seven minutes before reperfusion.

Further analysis: In additional pigs, samples of whole blood were taken 7, 27, 67 and 127 minutes after drug administration for analysis of the CsA concentration.

Paper III

The cardioprotective effect of EP 94 administration was examined in two different protocols containing 41 pigs. In protocol I, carried out in the closed-chest model, pigs were administered intravenous boluses of either vehicle or EP 94 (1 ug/kg and dose) after 5, 12, 19 and 26 minutes, or 26, 33 and 40 minutes of ischaemia. In protocol II, open-chest pigs were administered vehicle or EP 94 (0.2 µg/kg/minutes) through an intracoronary infusion into the jeopardized myocardium, started 30 minutes after ischaemia induction and maintained for 15 minutes.

Further analysis: Protein content of eNOS and phosphorylated eNOS was measured in the control and ischaemic/reperfused areas.

Paper IV

In order to further evaluate the cardioprotective effect demonstrated in paper III, a dose-finding study with administration of EP 94 at reperfusion was carried out. Using the closed-chest model, 34 pigs underwent the ischaemia and reperfusion procedure. After 26, 33 and 40 minutes of ischaemia the pigs were administered saline, or 0.2, 1, 5 or 25 µg/kg (at each dosing time) of EP 94.

Further analysis: mRNA and protein content of the different opioid receptor subtypes were measured with RT-PCR and immunoblots in the control and ischaemic/reperfused areas. Furthermore, immunohistochemical staining was performed to localize the opioid receptors in frozen sections of myocardial tissue, and blood concentration of EP 94 was measured.

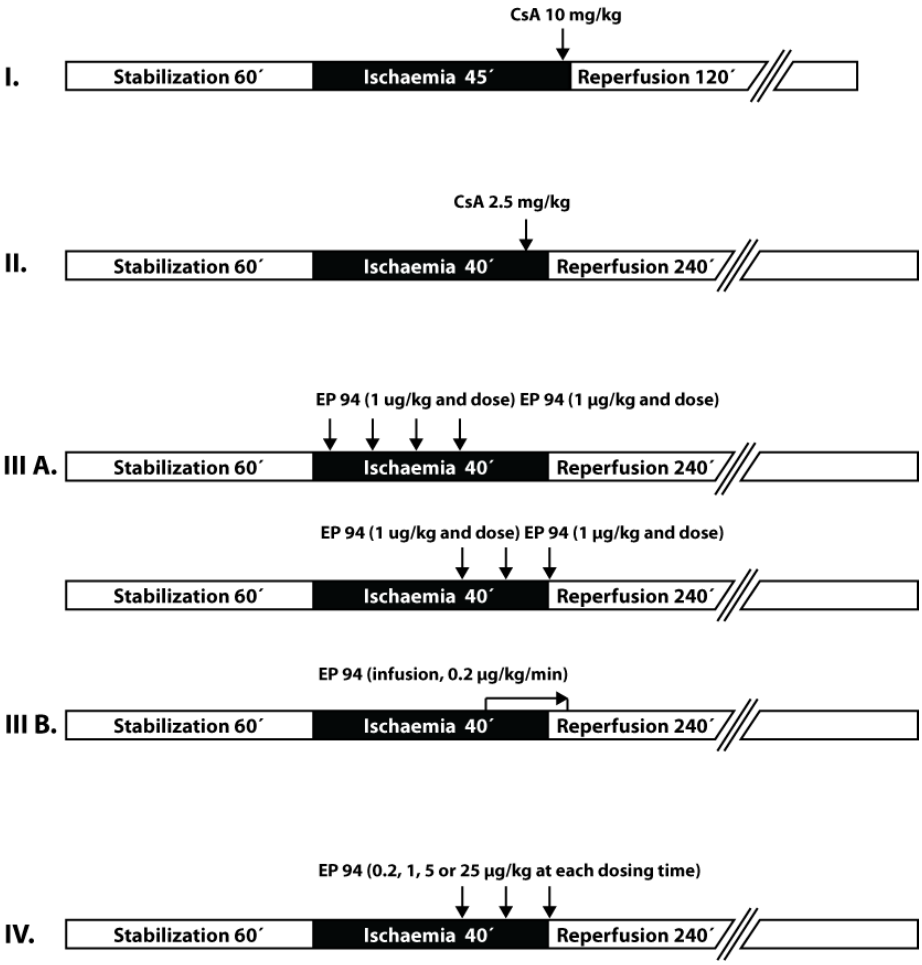

Figure 6. *Interventional protocols in the different studies.*

Statistical analysis

All values are presented as mean ± SEM, except for paper I where SD was used. In all tests, a $P < 0.05$ was considered statistically significant.

Comparison of infarct size: When only two groups were compared, an unpaired two-tailed Student's t-test of equal variance was used (papers I and II). When more than two groups were included in the

comparison, analysis of variance (ANOVA) with Dunnett´s post-hoc test was used (paper III). In the dose-response study (paper IV), linear regression was performed to evaluate a possible significant relationship between dose and infarct size reduction.

Haemodynamics: Differences in baseline characteristics and haemodynamic variables between groups were tested with repeated measures two-way ANOVA.

Interaction between CsA and Isoflurane: A two way ANOVA was used for analysis of infarct size and proapoptotic protein expression regarding possible interaction between the method of anaesthesia and CsA treatment.

mRNA and protein expression: When two groups were compared, an unpaired two-tailed Student's t-test of equal variance was used (papers I and III). Opioid receptor expression in the same individual (in the control and ischaemic/reperfused areas) was calculated with a paired two-tailed Student's t-test of equal variance.

RESULTS

Paper I. The cardioprotective effect of 10 mg/kg cyclosporine A

The cardioprotective potential of 10 mg/kg of CsA administered at reperfusion was investigated in 36 pigs undergoing the ischaemia and reperfusion procedure. Contrary to several studies in smaller animal models, CsA administration did not reduce infarct size in our open-chest porcine model. Furthermore, CsA induced an increased expression of the proapoptotic protein AIF. Because of the conflicting results in the first phase using isoflurane as general anaesthesia, the study was expanded with a second phase with the administration of pentobarbital. When analyzing the results in the different treatment groups, a cardioprotective effect with isoflurane administration compared with pentobarbital was noted (due to the reasons mentioned in the material and methods section this part of the study was not randomized) ($P < 0.05$). Furthermore, a possible negative interaction between isoflurane and CsA administration was observed, with increased expression of proapoptotic proteins, and a trend towards attenuation of infarct size reduction when combining the compounds.

Conclusion: 10 mg/kg CsA administered at reperfusion did not reduce lethal reperfusion injury in an open-chest porcine model. Whereas isoflurane confers cardioprotection, the data suggest a possible deleterious interaction between CsA and isoflurane with the possible induction of the apoptotic cascade.

Paper II. The cardioprotective effect of 2.5 mg/kg cyclosporine A

When studying the lower dosing regimen of 2.5 mg/kg CsA in 24 closed-chest pigs, no protection against lethal reperfusion injury was

observed. Furthermore, analysis of CsA blood concentration revealed lower levels than were reached in humans with the same dosing regimen.

Conclusion: Intervention with 2.5 mg/kg CsA at reperfusion did not reduce lethal reperfusion injury in a closed-chest porcine model.

Paper III. The cardioprotective effect of enkephalin analogue EP 94

In this paper, the cardioprotective effects of three different administration regimens with the novel enkephalin analogue EP 94 were investigated in a total number of 41 pigs. Two different porcine protocols of ischaemia and reperfusion were used: a closed-chest model attempting to resemble the clinical situation for acute myocardial infarction, and an open-chest model with the compound being infused distally to a coronary ligation. EP 94 reduced infarct size when administered both early and late during ischaemia in the closed-chest model ($P < 0.05$), as well as when infused into the ischaemic myocardium in the open-chest model ($P < 0.01$). Immunoblots revealed a possible mechanism for cardioprotection, with up-regulation of phosphorylated eNOS in relation to total eNOS in the pigs receiving EP 94.

Conclusion: The novel enkephalin analogue EP 94 reduced infarct size in two different porcine models of ischaemia and reperfusion. Increased phosphorylation of eNOS may be a part of the mechanism behind this cardioprotection.

Paper IV. Dose-dependent cardioprotection of EP 94 and opioid receptor expression in the porcine heart

In order to further investigate the potential cardioprotective effect of EP 94 we conducted a dose-finding study in our closed-chest porcine model. A total number of 34 pigs underwent the closed-chest ischaemia and reperfusion procedure, with administration of different doses of EP 94 at reperfusion. Infarct size measurements revealed a dose-response relationship between increasing doses of EP 94 and smaller infarct size ($P < 0.05$). mRNA for the κ- and δ-opioid receptors was detected, with a significant increase in ischaemic/reperfused areas, whereas the μ-opioid receptor was not detected. Immunoblots confirmed the existence of the κ- and δ-opioid receptors in the left ventricle, with immunohistochemical staining revealing expression by cardiomyocytes and vascular smooth muscle cells. Furthermore, an up-regulation of the δ-opioid receptor in ischaemic/reperfused areas was noticed ($P < 0.0001$), whereas the κ-opioid receptor expression remained unaffected by the ischaemic insult. Finally, a significant negative correlation between δ-opioid receptor mRNA expression and increased body weight was observed, and a significant difference in mean body weight compared with the pigs in study III ($P < 0.05$).

Conclusion: Enkephalin analogue EP 94 reduced infarct size in a dose-response manner in a closed-chest porcine model. The pig left ventricle expressed κ- and δ-opioid receptors, with an up-regulation of the δ-opioid receptor in ischaemic/reperfused areas. The μ-opioid receptor was not detected.

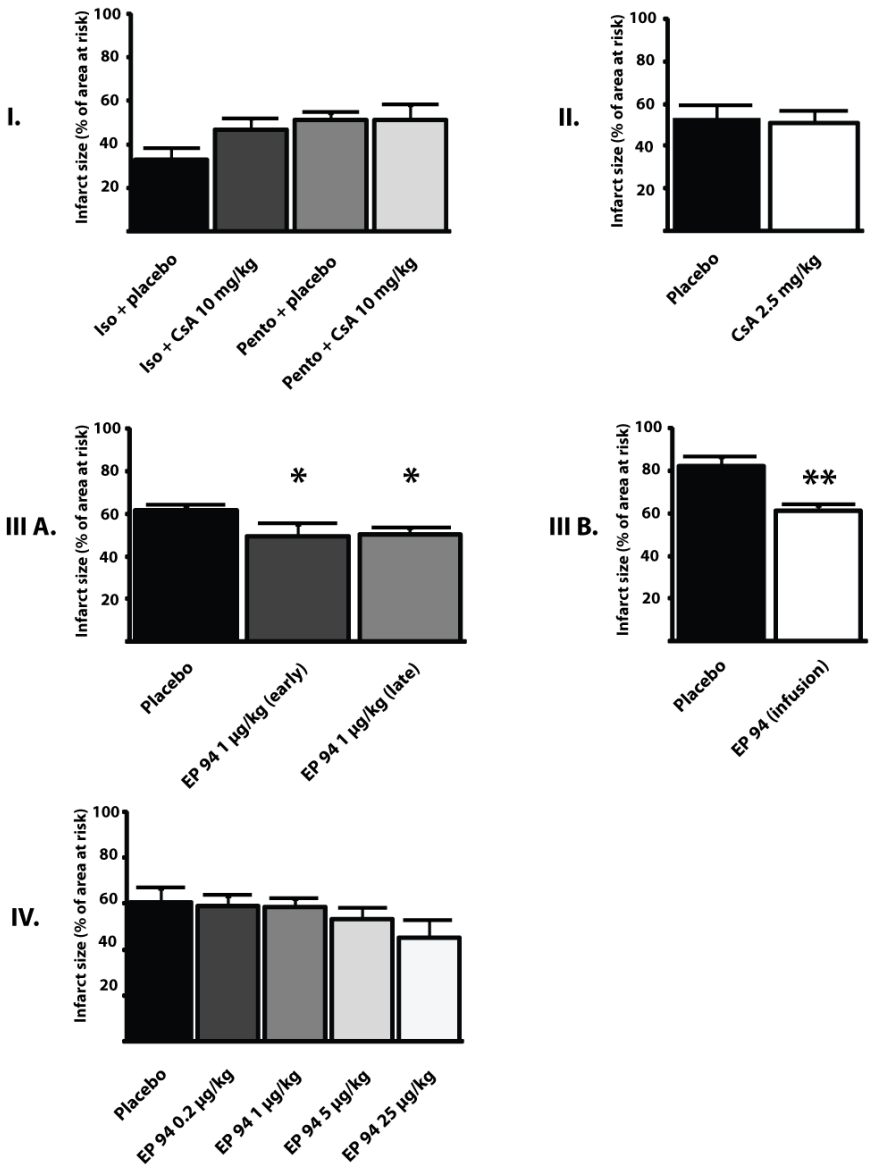

Figure 7. *Infarct size as a percentage of area at risk in the different studies. All data are presented as mean ± SEM. *P < 0.05; **P < 0.01.*

DISCUSSION

Since the initial description 50 years ago, myocardial reperfusion injury has attracted major research efforts. Despite this effort, however, no standard treatment strategy has thus far been established in the clinical setting. As outlined in the introduction section, the wealth of promising concepts presented in different experimental models have, in the vast majority of cases, turned out to be negative when translated to humans. In the present thesis, we investigated if two different compounds administered at reperfusion could reduce myocardial lethal reperfusion injury. These interventions were carried out in porcine models trying to mimic the clinical situation of acute myocardial ischaemia and reperfusion, in an attempt to confirm a possible beneficial effect in a large animal before translating the concepts to the clinical setting.

Cyclosporine A in reperfusion injury

First discovered in 1972, CsA has since been used as an immunosuppressant agent, primarily in the setting of organ transplantation. Over the last few decades, however, the compound has also been appreciated for its ability to limit lethal reperfusion injury[22, 30-33], possibly through inhibiting the opening of MPTP[29]. Even though the results are disparate, several animal studies using 10 mg/kg CsA at reperfusion have reported a reduction in infarct size[30, 136-137]. When our initial study (paper I) was carried out, this beneficial effect had only been investigated in smaller animal models, and the present study was therefore conducted in order to confirm a possible cardioprotective effect of 10 mg/kg CsA administered at reperfusion in a large animal model, before translating this concept to humans. The administration regimen, however, did not limit infarct size in our open-chest porcine model, a finding that has later been confirmed by other groups[138]. In

the initial phase of the study isoflurane was used as general anaesthesia. Even though well known for its ability to protect the heart against ischaemic insult[139-141], it has only recently been suggested that this effect is mediated through inhibition of MPTP opening[142-143]. This led us to suspect that cardioprotection mediated by CsA could be obscured by isoflurane treatment. We therefore repeated the CsA intervention procedure with pentobarbital anaesthetics, again without establishing any protective effect, but confirming earlier reports regarding cardioprotection with isoflurane administration. In order to address the possibility that CsA treatment would reduce apoptosis by means of MPTP closure after a period of permeability, proapoptotic proteins were analyzed in the ischaemic/reperfused areas. Surprisingly, the results revealed a possible induction of apoptosis with CsA treatment and, furthermore, a possible negative interaction between isoflurane and CsA treatment, a fact supported by larger infarct size with CsA treatment compared with vehicle. Although both compounds may target the MPTP, and reports exist that CsA attenuates the anaesthetic effect of isoflurane[144], the mechanisms behind this possible deleterious interaction remain to be elucidated.

When analyzing the results from the first study, Piot et al reported on the usage of 2.5 mg/kg CsA as an adjunctive therapy in patients presenting with acute ST-elevation infarction[135]. Although enrolling a limited number of patients, a significant reduction of both creatine kinase release and infarcted tissue measured by means of magnetic resonance imaging was noticed. This led us to consider that the dose used in paper I could have been too high, hence we conducted another series using 2.5 mg/kg CsA in a closed-chest model (paper II). This lower dose of CsA did not, however, confer cardioprotection in our model.

There are several possible explanations for the lack of effect with CsA administration in our studies. When analyzing the blood concentrations of CsA, species differences regarding pharmacokinetics of the drug are evident; administration of 2.5 mg/kg CsA in our porcine model

resulted in considerably lower concentrations compared with the clinical study. 10 mg/kg, on the other hand, resulted in higher concentrations, indicating that a dosage in the order of 5 mg/kg could reproduce the CsA blood concentrations reached in the clinical study and, possibly, cardioprotective effects encountered in humans, a fact supported by a small study in minipigs[145]. This would, however, indicate a very narrow therapeutic window for the compound, a possible troublesome fact in the clinical setting with patients presenting with different degrees of collateral flow and difficulties in predicting the exact time for reperfusion. Furthermore, reports indicate that CsA may mediate direct negative effects on the ischaemic myocardium, including modification of the cardiac energy metabolism, destabilization of hypoxia-inducible factor 1α, impaired endothelium-derived vasodilatation in coronary arteries, and deterioration of microvascular function[146-150]. Finally, one cannot exclude the fact that MPTP is not as crucial for the development of lethal reperfusion injury in the pig as has been advocated for other species.

In summary, CsA did not protect against lethal reperfusion injury in two different intervention protocols in the pig. Furthermore, the present study, as well as results obtained by other groups, indicates differences regarding CsA treatment between species, a fact that may be of great importance in translational programmes when developing pharmacological strategies for myocardial protection.

The cardioprotective effect of EP 94

As in the case with CsA, opioid peptides are used in the clinical setting for other reasons than their effects on the circulatory system. Despite this fact, the great importance for opioids in the heart, both during physiological regulation and different pathological disorders, is becoming increasingly evident. A substantial amount of data indicate different opioid peptides to be important players in the heart during

episodes of ischaemia and reperfusion[117], and several compounds targeting the opioid receptor subtypes have been shown to limit infarct size in experimental settings[117-120]. In analogy to CsA, most reports regarding opioids and their ability to reduce lethal reperfusion injury originate from smaller species, a premise that led us to investigate this concept in two different porcine models of ischaemia and reperfusion. In this setting, the novel enkephalin analogue EP 94 reduced myocardial infarct size when administered both during ischaemia and at reperfusion in a closed-chest model, as well as when infused into the jeopardized myocardium in an open-chest model (paper III). In order to further evaluate the cardioprotective effect of EP 94 we conducted a dose-finding study, confirming a beneficial effect of the intervention in a dose-response manner (paper IV), with the major protective effect observed in the highest dosage group. Interestingly, we were not able to repeat the cardioprotective effect from the earlier study with administration of 1 μg/kg. Comparison between the studies revealed a significant difference in mean body weight, with smaller animals in the latter study, indicating that variations in weight and age constitute a possible explanation for the differing results. This is supported by a study in rats reporting decreased expression of the μ-opioid receptor during heart development[111] and, furthermore, by our finding of a significant negative correlation between δ-opioid receptor mRNA expression and increased body weight.

EP 94 has main affinity for the μ- and δ-opioid receptors and, to a lesser extent, for the κ-opioid receptor. As we were unable to detect the μ-opioid receptor in the porcine heart, δ is the probable receptor responsible for the protection encountered in our models. This reasoning is further strengthened by the literature, where most reports regarding the beneficial effects of opioids in the setting of ischaemia and reperfusion attribute this to stimulation of the δ-subtype[117]. Even though we cannot exclude a possible remote effect of the given compound, the fact that an infusion into the jeopardized tissue

provided protective effects (paper III) indicates a direct action on local opioid receptors in the ischaemic myocardium.

As summarized in the introduction section, pathways responsible for the protective effects of opioids include, among others, the PI3K pathway, stimulation of PKC-δ, inhibition of glycogen synthase kinase-3β, opening of mitochondrial K(ATP) channels and inhibition of MPTP opening[115, 121-126]. Furthermore, eNOS is known to confer cardioprotection in a variety of species, including the pig[151-153], and other compounds targeting G-protein coupled receptors are known to increase the expression of eNOS[127-128]. We therefore addressed the possibility that eNOS could be a possible mediator of the beneficial effect observed in our porcine models. Although eNOS expression did not differ between the treatment and vehicle groups, phosphorylated eNOS (considered to be the biological active form) was significantly increased when expressed as a ratio of total eNOS after EP 94 administration. Even though several other possible mechanisms exist, and further studies are needed to confirm our findings, this represents one possible pathway for the cardioprotective effect seen with EP 94 administration. The difficulties with translating interventions from the experimental to the clinical setting, however, indicate that solely targeting one mechanism in order to reduce lethal reperfusion injury is probably not sufficient. It could, therefore, be advocated that an approach attempting to affect several different pathways, including pharmacological as well as mechanical interventions, would constitute an attractive alternative in order to reduce lethal reperfusion injury. An intervention in this context could, based on the results presented above, involve a compound targeting the opioid receptors.

Opioid receptors in the porcine heart

In addition to EP 94 intervention, we investigated the distribution of opioid receptor subtypes in the porcine left ventricle (paper IV). This

analysis demonstrated the existence of κ- and δ-opioid receptors, whereas the μ-opioid receptor was not detected, thus confirming results obtained in rat models[112-114]. This is, however, different from results obtained in humans, where μ- and δ-opioid receptors have been detected in both ventricles of the heart[110, 115-116], illustrating a possible difference between species and raising important questions regarding the translation of experimental data for opioid peptides to the clinical setting.

The alteration of opioid receptor expression in the setting of myocardial ischaemia and reperfusion is not well understood. To the best of our knowledge only one study in rats has addressed this issue, reporting an up-regulation of the κ-opioid receptor after a period of ischaemia[154]. In our study, a significant increase of the δ-opioid receptor expression was noticed in the ischaemic/reperfused area, whereas the κ-opioid receptor expression remained unaffected. mRNA for both the κ- and δ-opioid receptors was, however, significantly increased after a period of ischaemic insult. Even though further studies are needed to explore the precise regulation of opioids in the heart, both findings indicate opioid receptors as important players in the setting of myocardial ischaemia and reperfusion.

Methodological considerations

The study design in papers III and IV included a continuous infusion of morphine throughout the experiment; a possible mediator of cardioprotection in itself[155-156]. Commonly used as analgesia in the clinical setting of acute myocardial infarction, it is reassuring that the protective effect of EP 94 could be demonstrated on top of this treatment, indicating the compound as a promising cardioprotective agent. Of relevance throughout the thesis, all pigs in the four different protocols (papers I-IV) received acetylsalicylic acid and benzodiazepines. Whereas acetylsalicylic acid attenuates the

cardioprotective effects of ischaemic preconditioning, statins and morphine[157-159], the reports on benzodiazepines are conflicting: midazolam abrogates the protective effects of ischaemic preconditioning, while stimulation of the peripheral benzodiazepine receptor inhibits MPTP opening and limits infarct size[160-163]. Commonly used drugs to treat acute myocardial ischaemia, these possible interactions constitute important issues, both in our experimental models as well as in the clinical setting.

CONCLUSIONS

- Cyclosporine A does not reduce lethal reperfusion injury in porcine models, mimicking the clinical situation of ischaemia and reperfusion.
- The novel enkephalin analogue EP 94 confers cardioprotection in different porcine models, possibly through a NO-dependent mechanism.
- κ- and δ-opioid receptors are expressed in the left ventricle in the pig, whereas the μ-opiod receptor was not detected. δ-opioid receptor expression is increased after ischaemia and reperfusion.

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