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Metzsch, Carsten

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Department of Anesthesiology and Intensive Care
Institution of Clinical Sciences, Lund
Faculty of Medicine
Lund University, Sweden

MYOCARDIAL ISCHEMIA-REPERFUSION AND CARDIOPROTECTION

A study with microdialysis in a porcine model

Carsten Metzsch

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Carsten Metzsch, M.D.
Cardiothoracic Anesthesia and Intensive Care
Heart and Lung Division
Lund University Hospital
22185 Lund, Sweden.
carsten.metzsch@med.lu.se

This thesis is dedicated to my family

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Summary

The studies in this thesis are based on questions raised in the clinical setting.
Perioperative myocardial ischemia occurs more often than recognized. This may lead to myocardial infarction, increased morbidity, mortality, and health care costs.
In the first study, myocardial metabolism was investigated before, during, and after 30 min of regional coronary artery occlusion, utilizing the microdialysis technique, concomitantly with the monitoring of global circulation and local coronary artery flow in an open chest pig model. Myocardial interstitial metabolites demonstrated characteristic, significant, and reproducible changes as decreased glucose, increased glycerol, and increased lactate/pyruvate ratio during ischemia, normalizing after reperfusion. Of special interest was found that myocardial glycerol concentrations remained high initially at reperfusion, raising the hypothesis of this release corresponding to reperfusion injury. This model was used for the next two studies.
In cardiac surgery, episodes of myocardial ischemia or decreased myocardial performance are highly expected to occur. Patients with poor cardiac function will have a double benefit of an inotropic drug with anti-ischemic properties. Levosimendan may have this potential. In the second study, it was demonstrated that an infusion of levosimendan started before the coronary artery occlusion, as compared to start during the ischemia, reduced the effect of ischemia on the myocardial metabolism, improved, and preserved cardiac performance during this period.
In recent years, concerns with the use of perioperative beta-blockers have been debated. Beta-blockers may inhibit the pharmacological preconditioning elicited by volatile anaesthetics. In the third study, it was demonstrated that levosimendan, in the presence of beta-blockade, was still able to induce a cardioprotective effect on the myocardial ischemic metabolism.
During cold cardioplegic storage, or in the future during preservation of donor hearts by perfusion, monitoring of the donor heart before transplantation may be of benefit. We hypothesized, that myocardial microdialysate glycerol will reflect progressive damage. As the first step in pursuing this, in the fourth study, the course of myocardial metabolites was investigated during ten hours of cold cardioplegic storage. An accelerating myocardial glycerol accumulation was demonstrated during storage, after an initial stable period, probably reflecting the acceptable storage time.

Populärvetenskaplig sammanfattning

Studierna i denna avhandling är alla baserade på frågeställningar som uppkommit i den kliniska verksamheten.

I samband med narkos, kirurgi och omedelbart i efterförloppet kan det förekomma episoder med syrebrist i hjärtmuskeln (ischemi) som uppkommer vid hindrad blodtillförsel eller om blodtillförseln är otillräcklig för att tillfredsställa hjärtmuskelns krav på syre för utfört arbete. Detta förekommer sannolikt oftare än registrerat. Följden av sådana episoder kan bli hjärtinfarkt, hjärtsvikt, dödlighet och ökade kostnader för sjukvården. Särskilt patienter med hjärt- och kärlsjukdomar, diabetes och njursjukdom är i riskzonen.

Under hjärtischemi förändras ämnesomsättningen i muskeln karakteristiskt beroende på syrebristen, så kallad anaerob ämnesomsättning. Vissa ämnen, såsom mjölksyra och glycerol, ansamlas (ackumuleras) i vävnaden medan andra ämnen, som t.ex. pyrodruvsyra, visar en minskning. Ackumulationen av mjölksyra samt en ökning i kvoten mellan mjölksyra och pyrodruvsyra används för att upptäcka och beskriva graden av ischemisk ämnesomsättning och som uttryck för hur uttalad ischemin är.

Mikrodialys är en teknik med vilken man kan mäta olika substanser i en vävnad. En mikrodialyskateter kan vara uppbyggd enligt följande: i spetsen finns en tunn slang, som är sluten i ena änden, och väggen består av ett membran. Över membranet kan små substanser passera fritt (diffusion). Kopplad till denna spets finns två slangar, en tillförande och en avlägsnande. En pump kopplas till den tillförande slangen och tillför en vätska (perfusat). Vätskan som passerar membranet tar upp (dialyserar) olika substanser som finns i vävnaden där mikrodialyskateter är placerad. Vätskan (dialysatet) samlas sedan i små rör. Dessa rör byts med fastlagda intervall och innehållet analyseras. Mängden av substans som tas upp i dialysatet är direkt beroende på mängden i vävnaden, dvs. analysen speglar mängden i vävnaden vid tillfället när dialysatet samlades. På så sätt kan man följa förändringar i en vävnads ämnesomsättning över tid.

I delarbete I undersöktes ämnesomsättningen i hjärtmuskeln på gris med mikrodialysteknik. Vi mätte innan, under och efter 30 minuters tillfällig avstängning av ett mindre kranskärl. Avstängningen av kranskärlet gav ischemi i en mindre del av vänster hjärthalva. Mikrodialyskatetrar fanns placerade både i tillfälligt ischemisk och i normal hjärtmuskel. Hjärtfunktionen, cirkulationen och blodflödet i kranskärlet som tillfälligt avstängdes under ischemin övervakades under hela experimentet. Vi observerade typiska, uttalade, reproducerbara förändringar av substanserna i ischemisk vävnad och de förändringarna normaliserades efter återvänt blodflöde. Förändringar

som observerades var minskad nivå av glukos, ökad nivå av glycerol, mjölksyra och kvoten mjölksyra/pyrodruvsyra. Särskilt intressant var att glycerol låg kvar på en hög nivå i den tidigare ischemiska vävnaden under en kort period efter återvänt blodflöde trots normalisering av övriga substanser. Detta fenomen kan möjligen bero på ytterligare paradoxal vävnadsskada som kan förekomma när blodflödet återvänder. Hjärtat pumpade betydligt mindre minutvolym under en kort period vid återvänt blodflöde i motsats till små ändringar under den ischemiska perioden. Detta skedde utan samtidiga allvarliga hjärtrytmrubbningar. Detta visar paradoxen att hjärtat kan reagera negativt på återvänt blodflöde i ett ischemiskt område, även om området är en relativt begränsad del av vänster hjärthalva. I det tidigare avstängda kranskärlet ökade blodflödet betydligt utöver det normala för en period.

Den beskrivna modellen har använts i delarbete II och III, där olika läkemedels effekt på ämnesomsättningen i hjärtmuskeln under och efter ischemi utvärderats.

Djurförsök och resultat från studier med patienter i olika sammanhang, har visat att några få korta perioder med hjärtischemi före en efterföljande längre period med ischemi kan minska skadan på hjärtmuskeln, och leda till mindre utbredning av en eventuell hjärtinfarkt. Detta fenomen kallas ischemisk prekonditionering. Proceduren med tillfällig avstängning av kranskärl före en eventuell ischemisk episod är sällan praktiskt möjlig att genomföra i klinisk verksamhet och innebär sannolikt en risk i sig. Det krävs dessutom en förväntad period med efterföljande hjärtischemi eller en hög risk för detta för att proceduren ska ha ett syfte. Denna risk finns vid hjärtkirurgi, beroende på flera orsaker. Patienter som ska genomgå kranskärlskirurgi är definitivt kranskärlssjuka. Kirurgin är stor och innebär en påfrestning under och efter kirurgi med risk för hjärtischemi. Hjärtat och kranskärlen manipuleras direkt. Hjärtat skyddas med särskilt teknik under tiden hjärt-lungmaskinen är kopplad, men det finns risk för ischemiska episoder och skada ändå. Läkemedel som redan används i klinisk verksamhet kan ha förmåga att utlösa en reaktion i hjärtmuskeln som påminner om ischemisk prekonditionering. Ett sådant läkemedel är Levosimendan. Levosimendan används vid uttalad hjärtsvikt i syfte att öka mängden av blod som hjärtat pumpar ut per tidsenhet. Levosimendan förbättrar hjärtmuskelns pumpförmåga utan att kraftigt öka behovet av syretillförsel, till skillnad från flera andra läkemedel som används i samma syfte vid behandlingen av hjärtsvikt.

I delarbete II visade vi att en infusion av Levosimendan som påbörjas en kort stund innan hjärtischemi reducerar hjärtmuskelns anaeroba ämnesomsättning under ischemi och den typiska ansamlingen av substanser som normalt förekommer jämfört med om det påbörjas under den ischemiska perioden. Cirkulationen befanns också vara bättre under den ischemiska perioden i gruppen där Levosimendan påbörjades innan ischemin. Ingen skillnad fanns dock efter återvänt blodflöde. Vi drar slutsatsen att förbehandling påbörjad innan ischemin ger bättre effekt och skydd jämfört med behandling som påbörjas när ischemin uppträder. I samband med hjärtkirurgi finns

inte bara en risk för hjärtischemiska episoder utan också en risk för påverkad hjärtfunktion. För patienter med nedsatt hjärtfunktion som ska genomgå hjärtkirurgi kan behandling med Levosimendan möjligen ha en gynnsam och dubbelt förebyggande effekt om terapin påbörjas innan operationen. Både som en förbättring av cirkulationen innan försämring uppträder, och som ett skydd mot skada under ischemiska episoder.

Betablockerare är en grupp läkemedel som i stor utsträckning används vid hjärtsjukdomar, efter hjärtinfarkt, vid kronisk hjärtsvikt och som blodtryckssänkande medicin. Betablockerare har visat sig att ha en viss skyddande effekt i hjärtmuskeln under ischemi. Man anser också att betablockerare som används i samband med kirurgiska procedurer kan minska risken för hjärtinfarkt under och efter operation, särskilt hos vissa högrisk-patienter. Nyttoeffekten av detta har dock under senare år debatterats. Inhalation av narkosgas under kirurgi kan möjligen också skapa en effekt som motsvarar ischemisk prekonditionering. Några få studier visar att betablockerare sannolikt kan minska denna prekonditionerande effekt av narkosgas. Om betablockerare blockerar den skyddande effekten av Levosimendan kan det ha en viss klinisk betydelse, särskilt om Levosimendan är insatt delvis med ett skyddande syfte.

I delarbete III demonstrerade vi att förbehandling med Levosimendan hos grisar som också förbehandlades med en betablockerare, Metoprolol, fortsatt kunde utlösa en skyddande effekt på ämnesomsättningen i ischemisk hjärtmuskel.

Hjärtsvikt efter hjärttransplantation är inte ovanligt. Dödsfall inom 30 dagar efter hjärttransplantation förekommer i 5-10 % av fallen, och hjärtsvikt är orsaken till 30-40 % av dessa dödsfall. När donatorhjärtat förbereds, fram till dess att hjärtat transplanteras och pumpar acceptabelt finns det olika riskmoment som kan innebära skada. Den förberedande proceduren innan lagring är sammanfattningsvis att hjärtmuskelkontraktioner stannas med hjälp av en speciell, kall vätska och hjärtat kyls i syfte att kraftigt reducera ämnesomsättningen och behovet av syre och näringsämnen. Sedan lagras hjärtat i kall vätska. Kort lagringstid är viktigt för att hjärtat ska ha en god chans att fungera väl omedelbart efter transplantationen som vanligen genomförs inom några få timmar efter uttag av donatorhjärtat. Under tiden donatorhjärtat lagras i väntan på transplantation kan en praktisk metod för övervakning och bedömning av hjärtats överlevnadspotential ha betydelse. Den fortlöpande ansamlingen av glycerol i hjärtmuskeln under ischemi verkar som en markör för förlängd ischemi, och kan möjligen kopplas till progressiv irreversibel skada. Alltså kan detta vara en möjlig bedömningsmetod.

I delarbete IV studerades förloppet av substanser i hjärtats ämnesomsättning, med mikrodialysteknik, innan uttag av hjärta och sedan under tio timmars kall lagring. Glycerol i hjärtmuskeln visade ansamling under lagringstiden. Under de första

timmarna var det stabilt, men mot slutet av lagringstiden ökade ansamlingstakten. Detta motsvarar den kliniska kunskapen att upp till ca 4 timmar är en acceptabel lagringstid för ett donatorhjärta. Framtida studier kommer att visa om mängden av ackumulerat glycerol kan förutspå dålig hjärtfunktion efter transplantation. Om detta är fallet kan mikrodialystekniken få en plats i övervakningen och bedömningen av det donerade hjärta innan transplantation.

Original studies

This thesis is based on the following studies, referred to in the text by their respective Roman numerals (I-IV):

I.

Metzsch C, Liao Q, Steen S, Algotsson L.
Myocardial glycerol release, arrhythmias and hemodynamic instability during regional ischemia-reperfusion in an open chest pig model.
Acta Anaesthesiol Scand 2006; 50: 99-107.

II.

Metzsch C, Liao Q, Steen S, Algotsson L.
Levosimendan cardioprotection reduces the metabolic response during temporary regional coronary occlusion in an open chest pig model.
Acta Anaesthesiol Scand 2007; 51: 86-93.

III.

Metzsch C, Linnér R, Steen S, Liao Q, Algotsson L.
Levosimendan cardioprotection in acutely beta-1 adrenergic receptor blocked open chest pigs.
Acta Anaesthesiol Scand, accepted 2009, (in press)

IV.

Metzsch C, Steen S, Liao Q, Algotsson L.
Time dependent myocardial glycerol accumulation during cold cardioplegic storage.
(manuscript)

List of abbreviations

ANOVA	analysis of variance
ATP	adenosine-5'-triphosphate
CABG	coronary artery bypass grafting
CAD	coronary artery disease
CAF	coronary artery flow
CAO	coronary artery occlusion
CO	cardiac output
CPB	cardiopulmonary bypass
CVP	central venous pressure
dp/dt_{max}	maximum rate of systolic pressure development
ECG	electrocardiogram
FRM ANOVA	Friedman repeated measures ANOVA on ranks
G-3-P	glycerol-3-phosphate
HR	heart rate
i.m.	intramuscularly
i.v.	intravenously
KATP channels	ATP-sensitive potassium channels
LAD	left anterior descending coronary artery
LPR	lactate/pyruvate ratio
MAP	mean arterial pressure
min	minutes
NAD	nicotinamide adenine dinucleotide, oxidized
NADH	nicotinamide adenine dinucleotide, reduced
PA	mean pulmonary artery pressure
PMI	perioperative myocardial infarction
SAP	systolic arterial pressure
SD	standard deviation
SEM	standard error of the mean
SV	cardiac stroke volume
SVR	systemic vascular resistance
VF	ventricular fibrillation
VT	ventricular tachycardia

Preface

Perioperative myocardial ischemia probably occurs frequently, detected or undetected, varying from brief insignificant episodes to fatal infarctions. The complications of ischemia, with or without infarction, include arrhythmias, heart failure, patient discomfort, extended stay in intensive care units, increased health care costs and death, and thus have consequences for the patient as well as for health care.

On a daily basis, anaesthesiologists deal with the prevention, detection, and management of perioperative myocardial ischemia and its consequences. The phenomenon of myocardial ischemic preconditioning has demonstrated that it is possible to reduce the consequences of an ischemic insult. Drugs already in common use may influence and activate the preconditioning pathways.

The primary purpose of this thesis was to investigate temporary regional myocardial ischemia, the effects on myocardial metabolism and circulation, and pharmacological cardioprotection on these variables. The designs of the studies were based on questions raised in the clinical setting.

To secure functional recovery after transplantation, donor hearts need proper organ preservation and storage. Monitoring of the myocardial viability during storage may be useful.

The second purpose was to investigate the myocardial metabolism during prolonged cold cardioplegic storage, especially the course of myocardial glycerol, pursuing a hypothesis of a marker related to duration of storage, and perhaps to viability.

The common features of the studies in this thesis are myocardial ischemia, cardioprotection, and microdialysis.

To be - or not to be anesthesiologist no. 7,
- that's the question!

INTRODUCTION

- so habe ich den neuen Ausdruck der Ischämie vorgeschlagen,
um damit die Hemmung der Blutzufuhr,
die Vermehrung der Widerstände des Einströmens zu bezeichnen.
R Virchow, Berlin, 1858.

Myocardial ischemia

Myocardial ischemia is generally defined and characterized by a blood flow insufficient to supply the oxygen and nutrients demanded for the present functional and metabolic needs of the myocardium. Myocardial ischemia has dramatic effects on contractile performance and cellular metabolism.

In swine, gradually decreasing coronary artery blood flow decreased the performance of the affected myocardium and induced a graded lactate release (Guth 1990). When severe ischemia occurs, mechanical dysfunction quickly follows in seconds to minutes ([1]Jennings 1991, Stanley 1997). Metabolism is affected at several steps. Complete glucose and fatty acid oxidation are inhibited as mitochondrial respiration ceases quickly. Glycogenolysis and anaerobic glycolysis with production of ATP continue for a while ([1]Jennings 1991, Stanley 1997). This is ultimately inhibited by lactate accumulation, acidosis, or NADH (Neely 1981, Cross 1995). Progressive inhibition of the metabolic pathways, by the lack of substrates, by the inhibition from end-products, and acidosis, leads to the accumulation of intermediary metabolites and degradation products. This includes nucleoside intermediates, glucose-6-phosphate, glucose-1-phosphate, glycerol-3-phosphate (G-3-P), and glycerol, in addition to lactate, H^+, and NADH as mentioned ([1]Jennings 1991, Ye 1996). Also intermediates of fatty acid metabolism accumulate (Moore 1980, Neely 1981). In myocardial tissue, metabolic markers of ischemia are initially decreasing levels of creatine phosphate and ATP, followed by increasing **lactate**, whereas accumulating **glycerol** may reflect prolonged ischemia (Ye 1996). An important regulator of cellular metabolism is the cell redox state, symbolized by the NAD/NADH ratio. This indicates the metabolic abilities to oxidize substrates and metabolites, including a continued anaerobic glycolytic production of ATP. The more available NAD, the more active the oxidative potential. Normally, most produced NADH is oxidized back to NAD by oxidative pathways in mitochondria dependent on oxygen supply. A minor supply of NAD can also be regenerated in the process of conversion of pyruvate to lactate by the simultaneous conversion of NADH through the lactate dehydrogenase reaction.

The NAD/NADH ratio is inversely proportional to the tissue lactate/pyruvate ratio, LPR, (Luft 2001). This supports that the **lactate/pyruvate ratio** is a detector and a marker of the degree of myocardial ischemia. In isolated rat hearts, where the lactate/pyruvate ratio was artificially manipulated by adding either lactate or pyruvate to the glucose-based perfusate during low-flow ischemia, the post-ischemic functional recovery in the lactate perfused hearts was severely reduced as compared to the control and to the pyruvate perfused hearts (Cross 1995). This happened despite that the pyruvate perfused hearts had a lower intracellular pH during ischemia, the same lactate concentration, and a higher lactate production during ischemia (Cross 1995). It was concluded that a high lactate/pyruvate ratio corresponding to a high NADH/NAD ratio (or inversely to NAD/NADH), inhibited anaerobic glycolysis, affecting the cells ability to recovery (Cross 1995).

With severe ischemia, cardiomyocyte cell death begins within 20 to 60 minutes after the onset and continues to nearly all cells in the ischemic area within 6 hours ([1]Jennings 1991). This occurs progressively as a "wavefront phenomenon" (Reimer 1977). Collateral flow may slow this progression (Reimer 1979), the rate of anaerobic glycolysis and the metabolite accumulation ([1]Jennings 1991). Ischemia with permanent occlusion leads to infarction, but repeated brief episodes of myocardial ischemia may also lead to infarction (Geft 1982). One of the characteristic features associated with the transition from potential reversible to irreversible ischemic injury is high tissue levels of metabolites such as glucose intermediates, lactate, G-3-P, and cessation of anaerobic glycolysis ([1]Jennings 1991).

Myocardial ischemia and infarction may induce serious ventricular arrhythmias early within the first hour of ischemia (Perron 2005). During myocardial ischemia, concentrations of catecholamines may rise in the ischemic tissue (Lameris 2000). This may impose a risk of further damage (Opie 1975, Kübler 1994).

At reperfusion, aerobic metabolism is restored in viable myocardium ([1]Jennings 1991, Stanley 1997). The restoration of ATP levels is delayed ([1]Jennings 1991). The accumulated metabolites are washed out or metabolized ([1]Jennings 1991). An increased rate of fatty acid oxidation compared to pyruvate oxidation exists initially (Stanley 1997). Reperfusion within a few hours may preserve and salvage still viable cardiomyocytes (Reimer 1977). Reversible injured myocardium may demonstrate a variable period of contractile dysfunction after reperfusion termed "myocardial stunning" ([1]Jennings 1991, Kloner 1991, Ferrari 1996). Reperfusion itself, necessary for major myocardial salvage, may paradoxically induce injury of not yet irreversible ischemic injured cells, "reperfusion injury" (Monassier 2008), and reperfusion arrhythmias may occur (Perron 2005).

Perioperative myocardial injury

Perioperative myocardial ischemia and infarction probably occur more often than recognized. Reported incidence varies depending on the surgical setting and the presence of preoperative risk-factors in the selected group of patients, the methods of detecting myocardial ischemia, and the dynamically changing definitions and diagnostic criteria for myocardial infarction (Priebe 2004, Priebe 2005). In noncardiac surgery, some of the identified preoperative risk factors for perioperative cardiac complications including myocardial infarction are: high-risk surgery (major vascular, intraperitoneal, intrathoracic), ischemic heart disease, congestive heart failure, cerebrovascular disease, insulin therapy and high serum creatinine (Lee 1999).

In men, with or at risk of coronary artery disease (CAD) undergoing elective noncardiac surgery, Mangano found an incidence of intraoperative ischemic ECG-changes of 25% and an incidence of postoperative ischemic ECG-changes of 41% (Mangano 1990). 11 of 12 patients with myocardial infarctions did have postoperative ischemic episodes (Mangano 1990).

Episodes of perioperative myocardial ischemia increase the risk of myocardial infarction and negative outcome ([1]Landesberg 2003, [2]Landesberg 2003, Priebe 2004). Furthermore, the duration of ischemic perioperative ECG changes correlates to cardiac troponin I release (Landesberg 2001). Perioperative myocardial infarction (PMI) is associated with increased short- and long-term mortality (Priebe 2005).

Most patients surviving perioperative myocardial infarctions have angiographically extensive CAD (Priebe 2005). Some cases of PMI seem to be caused by plaque haemorrhage, rupture, and thrombus formation (Priebe 2004, Priebe 2005). However, PMI is mostly of the non-Q-wave type, preceded by ST-segment depressions, and may be associated with episodes of tachycardia (Landesberg 2001, [2]Landesberg 2003, Priebe 2004, Priebe 2005). This suggests that prolonged ischemia caused by supply-demand imbalance is of importance.

Studies of perioperative cardiac enzyme release in noncardiac surgery indicate that all grades of myocardial injury probably exist. The level of this release is associated with poor outcome ([1]Landesberg 2003, Kim 2002, Oscarsson 2004, Howell 2004).

During CABG surgery perioperative episodes of myocardial ischemia may occur in 47% of cases before cardiopulmonary bypass (CPB), and in 63% within 8 hours after revascularization (Tupper-Carey 2000). The occurrence of ischemic episodes was associated with increased postoperative levels of troponin I, even in the absence of diagnosed myocardial infarction (Tupper-Carey 2000). Perioperative myocardial injury after cardiac surgery detected by increased release of cardiac enzymes is also associated with a higher mortality (Steuer 2002, Kathiresan 2004). The mortality may correlate in a dose-responsive way to the magnitude of troponin I release (Croal 2006).

Among many strategies applied to the prevention or protection against myocardial ischemia in the perioperative setting are "pharmacological preconditioning" and the use of beta-blockers.

Myocardial preconditioning

The first description of the phenomenon of myocardial ischemic preconditioning is often referred to 1986. Murry, Jennings, and Reimer demonstrated in dogs that four 5 min cycles of circumflex artery occlusion interspersed with 5 min of reperfusion before 40 min of sustained ischemia reduced the subsequent infarct size to 7.3% of the myocardial area at risk as compared to 29.4% in animals not preconditioned (Murry 1986). There was no effect of ischemic preconditioning if the sustained ischemic period was extended to 3 hours (Murry 1986). Earlier, it has been demonstrated in rats, that myocardial necrosis in one region will induce protection in previously undamaged myocardium against injury by toxic doses of isoproterenol one week after the necrosis was induced (Dusek 1970). The phenomenon was termed "myocardial resistance". Anatomically reduced infarct size after ischemic preconditioning by protocol in the naive human myocardium not destined to undergo any procedure has (for obvious reasons) not been demonstrated. Some documentation exists that the human myocardium can be preconditioned (Rezkalla 2007). Studies with indirect indices on reduced injury have been performed. One example is a study in CABG surgery patients (Jenkins 1997). After the initiation of CPB, aortic cross-clamping was applied for 2 periods of 3 min duration separated by 2 min of reperfusion before cross-clamping and induced ventricular fibrillation during graft anastomosis. The postoperative release of troponin T was reduced in the preconditioning group as compared to control patients (Jenkins 1997).

A characteristic feature of ischemic preconditioning is the memory. After the preconditioning stimulus, the duration of protection is about 1-3 hours, "classic ischemic preconditioning". Thereafter protective capabilities are lost, but protection reappears without a new stimulus at about 12-24 hours and lasts to about 3-4 days after the preconditioning stimulus, "second window of protection" (Yellon 2003, Zaugg 2003). Intensive research has been performed on the signalling pathways. Several triggers, mediators, and effectors have been identified (Yellon 2003, Zaugg 2003, Downey 2007). The entire mechanism, or probably several pathways, has not been fully elucidated. One important key seems to be the ATP-sensitive potassium channels, KATP channels (Gross 2003, Yellon 2003, Zaugg 2003, Downey 2007). Experiments with different activators and inhibitors of these channels have demonstrated their role in inducing myocardial preconditioning when activated (Gross 2003, Yellon 2003). They exist as sarcolemmal channels and as inner mitochondrial membrane channels (Gross 2003, Yellon 2003). They are found in the myocardium as well as in coronary and systemic vessels (Toller 2006). The channels

are ATP-sensitive and are inhibited by physiological concentrations of ATP (Yellon 2003). Some important pathways, by which the opening of KATP channels induces protection to the myocardium, are probably interactions with protein kinase C, reactive oxygen species, and inhibition of the mitochondrial permeability transition pore (Yellon 2003, Zaugg 2003, Downey 2007, Hausenloy 2009). The last is supposed to be an end-effector of cell death (Monassier 2008).

The KATP channels are also involved in the preconditioning offered by volatile anaesthetics (Bienengraeber 2005), and probably in the phenomenon of "remote preconditioning" whereby the myocardial tissue is protected against injury during ischemia-reperfusion following short preconditioning ischemic periods in other "remote" organs as e.g. skeletal muscle (Kanoria 2007).

In addition to the effect of reduced or delayed ischemic myocardial injury, there are also indications that preconditioning may reduce myocardial stunning after ischemia, as demonstrated in experimental animals (Shizukuda 1993, Urabe 1993). Preconditioning may reduce reperfusion arrhythmias (Yellon 2003), also in humans as demonstrated in CABG patients. An ischemic preconditioning protocol by aortic cross-clamping was applied, and the incidence of postoperative ventricular arrhythmias was reduced in the preconditioned group as compared to control patients (Wu 2002).

Ischemic preconditioning affects the myocardial metabolism during the subsequent index ischemia generally by slowing down myocardial ischemic metabolism as compared to non-preconditioned ischemic control. This has been demonstrated in experimental animals as reduced anaerobic glycolysis with reduced lactate accumulation (Murry 1990, [2]Jennings 1991, Van Wylen 1994, [2]Wikström 1995, Vogt 2002), reduced accumulation of glucose intermediates and G-3-P ([2]Jennings 1991), and reduced ATP degradation (Murry 1990, [2]Jennings 1991).

Preconditioning by ischemic protocols or by activation of the involved pathways with drugs, "pharmacological preconditioning", may delay injury and protect the human myocardium against injury during ischemia and reperfusion, however, properly timed reperfusion is obligate to major salvage (Kloner 2004). An important part of the preconditioning effect is probably exerted at reperfusion (Downey 2007, Hausenloy 2007). *As preconditioning protocols involving repeated temporary myocardial ischemia in the clinical setting are not possible or likely unsafe to perform, the concept of pharmacological preconditioning is more appealing.*

Levosimendan

Levosimendan is an inotropic drug for use in heart failure. Early clinical randomized trials indicated that levosimendan beside clinical improvement may actually decrease mortality of cardiac failure. In the RUSSLAN study (Moiseyev 2002), patients with left ventricular failure complicating acute myocardial infarction were randomized to

different infusion doses of levosimendan or placebo. There was an overall reduced mortality at 14 and 180 days after treatment with the drug as compared to placebo (Moiseyev 2002). In the LIDO study (Follath 2002), patients with low output heart failure in different clinical settings were randomized to levosimendan or dobutamine. There was a reduced mortality at 180 days after treatment with levosimendan as compared to dobutamine (Follath 2002). However, more recent trials have not confirmed these beneficial effects of levosimendan on survival in heart failure. Another issue with levosimendan that in the recent years has attracted a growing interest is the potential anti-ischemic effects.

The effects of levosimendan on hemodynamics, as increased contractility, improved ejection fraction, increased cardiac output, reduced cardiac filling pressures, and reduced systemic, pulmonary and coronary vascular resistance, are based on its positive inotropic, lusitropic, and vasodilatatory properties (Papp 2005, Toller 2006, Lehtonen 2007). Levosimendan is a calcium sensitizer. It enhances myocardial contractility by binding to troponin C, stabilizing the binding of calcium and prolonging the systolic contractile interaction between actin and myosin (Toller 2006). The binding of levosimendan to troponin C is calcium concentration dependent, and does not affect diastolic function (Toller 2006). At high concentrations (>0.3 µM; >100ng/ml) levosimendan probably has a dose-dependent phosphodiesterase-III-inhibitory effect (Toller 2006), with heart rate increase as a consequence. Levosimendan has an active metabolite OR-1896 that can prolong the clinical effects for several days after ending a 24 h infusion (Lehtonen 2007, Lilleberg 2007).

Levosimendan has also demonstrated effects on myocardial stunning (Toller 2006). In patients with acute coronary syndrome undergoing angioplasty, a loading dose of levosimendan 10 min after the procedure improved the performance of stunned myocardium as compared to placebo (Sonntag 2004). Levosimendan may slightly prolong the QT, but it is not yet clarified whether levosimendan has an arrhythmogenic potential, or the contrary, as conflicting results have been presented (Lehtonen 2007).

In patients undergoing cardiac surgery, levosimendan has also demonstrated ability to improve cardiac performance after cardiopulmonary bypass (Lilleberg 1998, Nijhawan 1999). Of special interest is that levosimendan administered after CPB was able to significantly increase cardiac output without a significant increase in myocardial oxygen consumption or myocardial lactate production (Lilleberg 1998).

Levosimendan activates KATP channels. This contributes to vasodilatation and probably anti-ischemic effects (Kopustinskiene 2004, Papp 2005, Toller 2006). The anti-ischemic properties of levosimendan have been investigated, but not with consistently positive results (Papp 2005, Toller 2006).

In isolated rabbit hearts during 120 min of ischemia by occlusion of a circumflex artery branch, levosimendan started 30 min after occlusion reduced the intensity of

ischemia measured with epicardial NADH-fluorescence as compared to control (Rump 1994).

In isolated guinea pig hearts with levosimendan in the perfusate during 40 min of global low-flow ischemia, the tissue lactate accumulation was reduced and ATP was spared as compared to control (du Toit 1999).

In isolated rabbit hearts, two short preconditioning periods with levosimendan in the perfusate before 30 min of global myocardial ischemia and 120 min of reperfusion reduced the infarct size, and improved post-ischemic cardiac performance as compared to control (Lepran 2006).

In dogs, levosimendan started before 60 min of ischemia by occlusion of the left anterior descending artery (LAD) and 3h of reperfusion reduced the infarct size and increased coronary collateral flow as compared to control (Kersten 2000). The effects were blocked with the KATP channel blocker glyburide (Kersten 2000).

In pigs, during 30 min of coronary artery occlusion and 30 min of reperfusion, levosimendan initiated in advance of ischemia improved global cardiac performance and coronary flow in the peripheral ischemic zone as compared to control (du Toit 2001). No difference versus control was found on the myocardial tissue concentrations of ATP, and there were an increased number of arrhythmias in the levosimendan group (du Toit 2001).

In pigs, during regional low-flow ischemia by constriction of the LAD impairing regional contractility, increasing doses of levosimendan initiated approximately 45 min after start of ischemia improved global performance, had no effects on myocardial oxygen consumption in the ischemic myocardium, but negatively affected performance and increased lactate release in the ischemic myocardium (Tassani 2002).

In patients undergoing CABG surgery, a loading dose of levosimendan before CPB improved postoperative cardiac performance and reduced the postoperative troponin I release as compared to placebo (Tritapepe 2009).

In patients with low ejection fraction undergoing cardiac surgery, there may be a double benefit by initiation of levosimendan before surgery. First, the circulation may be optimized before hemodynamic deterioration occurs; and second, the pharmacological preconditioning of the myocardium may attenuate the consequences of perioperative myocardial ischemic injury. The optimal timing and dosing of levosimendan as an anti-ischemic agent is not thoroughly investigated.

Beta-adrenergic receptor antagonists

The pathophysiology during the perioperative period includes a neuroendocrine stress response with increased sympathetic activity and increased levels of circulating catecholamines. Beta-adrenergic receptor antagonists (beta-blockers) may affect this perioperative response as well as have direct myocardial anti-ischemic properties.

In dogs, propranolol pretreatment before circumflex coronary artery occlusion reduced the infarct size at 24 hours as compared to control, and the infarct size was reduced more than with propranolol started after occlusion (Rasmussen 1977). In rabbits, celiprolol infusion before, during and shortly after 30 min of regional myocardial ischemia reduced the infarct size at 2 days after reperfusion compared to control (Chen 2007). In pigs, intravenous acebutolol given during severe ischemia, by LAD constriction, increased coronary blood flow to the ischemic myocardium, improved regional myocardial function, and decreased coronary vein lactate release compared to control (Coetzee 2002). In pigs, intravenous metoprolol administered early during 90 min of LAD occlusion resulted in a smaller infarct size and improved recovery of left ventricular ejection fraction several days after reperfusion compared to placebo (Ibanez 2007).

In patients with or at risk of CAD undergoing noncardiac surgery, perioperative prophylactic atenolol started 30 min before surgery, reduced the incidence of postoperative myocardial ischemic episodes during the first 2 days by 50%, from 34% in the placebo group to 17% in the atenolol group. The mean perioperative heart rate was lower in the atenolol group. Atenolol reduced the two years mortality rate from 21% to 10% for patients surviving to after discharge (Mangano 1996+Wallace 1998). In high cardiac risk patients undergoing vascular surgery, perioperative bisoprolol started more than one week before surgery and continued to 30 days postoperatively reduced the combined risk of perioperative cardiac death and myocardial infarction within 30 days from 34% to 3.4% compared to placebo, and the mean perioperative heart rate was lower with bisoprolol treatment (Poldermans 1999). In vascular surgery, patients on higher doses of beta-blockers and with lower heart rates had a reduced incidence of perioperative myocardial ischemia and release of troponin T, and a decreased post discharge mortality and incidence of myocardial infarction during follow-up (Feringa 2006). One meta-analysis comprising ten randomized studies including 2176 patients undergoing noncardiac surgery concluded that studies where perioperative beta-blockade achieved most effective heart rate control demonstrated a reduced incidence of postoperative myocardial infarction (Beattie 2008). In the POISE multicenter study comprising more than 8000 patients undergoing noncardiac surgery and receiving perioperative metoprolol up to 30 days postoperatively or placebo, the use of beta-blocker reduced the incidence of myocardial infarction (Devereaux 2008).

In CABG surgery, patients preoperatively on chronic beta-blocker therapy have reduced 30-day mortality (Ferguson 2002, ten Broecke 2003). In one meta-analysis on twenty studies with 778 patients undergoing cardiac surgery, the use of perioperative esmolol demonstrated a reduced incidence of perioperative myocardial ischemia and arrhythmias, however, the incidence of myocardial infarction and mortality was unaltered ([1]Zangrillo 2009).

Some concerns may exist with the use of perioperative beta-blockers as highlighted by the POISE study mentioned above, where the overall mortality and the incidence of

stroke was higher in the beta-blocker group, despite the reduced incidence of myocardial infarction (Devereaux 2008). In the subgroup of patients with a left ventricular ejection fraction of less than 30% undergoing CABG surgery and preoperatively on beta-blockers, there may be a higher mortality, despite the overall reduced mortality benefit for all patients (Ferguson 2002).

Interestingly, in rat hearts, the stimulation of beta-1 adrenergic receptors shortly before 40 min of global ischemia and 30 min of reperfusion produced a protective effect on myocardial performance and reduced creatine kinase release at reperfusion as compared to control, and the effect was comparable to the effect of ischemic preconditioning (Frances 2003). In rabbits, with 30 min of regional myocardial ischemia and 3 hours of reperfusion, it has been demonstrated that esmolol and metoprolol have the capability to inhibit the infarct size reducing effect of preconditioning by volatile anaesthetics (Lange 2006, Lange 2008). However, it is indicated that beta-1 adrenergic signalling is not essential for ischemic preconditioning (Iliodromitis 2004, Lange 2006). It is possible that the beta-1 adrenergic receptors have a dual role as a potential participant in the mediation of ischemic damage as well as in preconditioning (Spear 2007), dependent on the timing of activation or inhibition. *It may be of clinical interest, and concern, whether beta-blockers have a protective effect or a potential to block the protection offered by other agents.*

Heart preservation

When a donor heart is harvested and stored for transplantation it fulfils the criteria for myocardial ischemia. Different techniques are used to significantly reduce the demands of the myocardium to preserve the metabolic and functional capacities of the heart to ensure adequate recovery at transplantation. In general, a modified version of cold cardioplegia used in other types of cardiac surgery is adopted. The three major components of cold cardioplegic storage are: the interruption of contractions, the induction and continuance of hypothermia, and different additives in the cardioplegic solution to affect metabolic or structural changes (Hearse 1980). With this generally applied method, there are some limitations as donor heart ischemic time continues to be a predictor of mortality (Taylor 2007, Taylor 2008). Poor or no functional recovery of the donor heart is not uncommon. After heart transplantation, the thirty-day mortality is about 8% (Luckraz 2005) with early graft failure in about 30-40% of these cases (Luckraz 2005, Taylor 2007, Taylor 2008).

During the cold storage, anaerobic metabolism continues at a low rate with breakdown of high energy phosphates and accumulation of metabolites (Humphrey 1991, Jamieson 2008). Mitochondria successively lose metabolic capacity and integrity (See 1992). Animal experiments have indicated that myocardial tissue contents of high energy phosphates at reperfusion may correlate to the functional

recovery of the heart (Humphrey 1991, Carteaux 1994). Monitoring during preservation and storage predictive for viability and recovery may be of benefit. This may be useful if using marginal donors, or as monitoring during continuous perfusion techniques, and when investigating extension of the storage time. Multiple sequential biopsies or nuclear magnetic resonance spectroscopy for the evaluation of myocardial viability during storage may induce risks and logistic difficulties in the clinical setting. Microdialysis may offer a potential to evaluate the viability of organs during harvest and preservation before transplantation (Jamieson 2008). The monitoring may be extended into the post-transplantation period. During cold preservation of pig livers for up to 15 hours, there was a progressive increase of tissue glycerol concentrations (Nowak 2003). Pig livers stored cold for 24 hours after preservation with different solutions demonstrated increasing tissue glycerol concentrations that may correlate to liver enzyme release and other signs of tissue injury (Puhl 2006). In pigs during renal ischemia, tissue glycerol increased with the duration of ischemia, and a concentration cut-off was identified that was associated with impaired post-ischemic renal function (Weld 2008).

As glycerol may be a better marker than lactate of prolonged myocardial ischemia (Ye 1996), *microdialysate glycerol concentration may have a potential in monitoring of the myocardium during prolonged cold ischemic storage.*
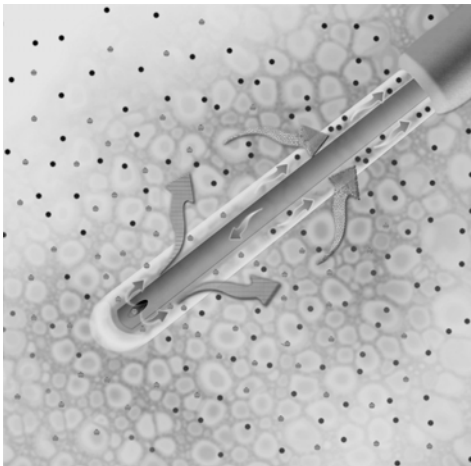

Cardiac microdialysis

With the microdialysis technique, tissue metabolism can be investigated semi-continuously in vivo. A microdialysis catheter, or "probe", consists primarily of a semipermeable dialysis membrane surrounding an internal lumen with inlet and outlet tubing. The microdialysis catheter is implanted into the target tissue and connected with inlet tubing to a microperfusion pump. The pump delivers a "perfusate". Metabolites or substances in the tissue interstitial space will, depending on the concentration gradients between the interstitial fluid and the perfusate, diffuse over the membrane and be recovered with the "dialysate" or "microdialysate" through outlet tubing to sampling vials. The dialysate may then be analysed for concentrations of the investigated substances or metabolites. The principle is demonstrated in the figure.

The quantity of the diffusion to the dialysate, "recovery", is dependent on characteristics of the tissue as temperature and complexity, characteristics of the dialysis membrane as length and pore size, the perfusate composition and flow rate, and the substance itself (Plock 2005). By choosing the composition of the perfusate, the perfusate flow rate, the sampling interval, and the dialysis membrane length, size and pore characteristics, an optimized microdialysis experiment setup may be achieved.

An important characteristic of the microdialysis experiment is the extraction fraction, or "relative recovery" of the investigated metabolite or substance (Plock 2005). This is the concentration of the metabolite in the dialysate in relation to the true concentration in the tissue interstitium surrounding the dialysis membrane. This means that if no calibration to the tissue concentration is done, the microdialysis experiment investigates concentration trends and not absolute concentrations. This is acceptable in experiments designed to investigate changes during a manipulation of tissue metabolism.

The validity of myocardial microdialysis has been tested in an open chest pig model of myocardial ischemia. Sampling from ischemic and non-ischemic myocardium was performed with the CMA20 microdialysis catheter and a perfusate flow of 2 µl/min. Microdialysate concentrations of lactate and ATP degradation metabolites obtained from the ischemic and the non-ischemic myocardium correlated strongly to the respective tissue concentrations in biopsies at the same time ([1]Kavianipour 2003).

Microdialysis used in the investigation of ischemic preconditioning in the porcine myocardium has demonstrated reduced lactate accumulation during ischemia as compared to non-preconditioned tissue ([2]Wikström 1995, [2]Kavianipour 2003).

Courtesy of CMA Microdialysis.

THE STUDIES

Aims of the studies

I.

To investigate myocardial metabolism during temporary regional coronary artery occlusion and subsequent reperfusion, utilizing the microdialysis technique, concomitantly with the monitoring of global and local circulatory changes.

II.

To investigate the cardioprotective effects of levosimendan started before ischemia-reperfusion, as compared to levosimendan started during coronary artery occlusion, on the myocardial ischemic metabolism and on the circulation.

III.

To investigate if levosimendan, in the presence of beta-1 adrenergic receptor antagonism with metoprolol, can induce a cardioprotective effect on the myocardial ischemic metabolism.

IV.

To investigate the course of myocardial glycerol accumulation and glycolytic metabolites during ten hours of cold cardioplegic storage using microdialysis.

Methods

In brief

I:

The myocardial metabolism was studied with microdialysis during myocardial ischemia and reperfusion concomitantly with the monitoring of the circulation and local coronary artery flow in an open chest pig model. Microdialysis catheters were placed in the ischemic myocardium and in non-ischemic control myocardium. A regional coronary artery was occluded for 30 min. After reperfusion the observation continued for 180 min.

II:

The effects of levosimendan started before ischemia was compared to the effects of levosimendan started during coronary artery occlusion on myocardial ischemic metabolism and on the circulation. Microdialysis catheters were placed in the ischemic myocardium and in non-ischemic control myocardium. A regional coronary artery was occluded for 30 min. After reperfusion the observation continued for 120 min. The animals were divided into two groups, a group where levosimendan was started before the coronary artery occlusion (protection), and a group where levosimendan was started 10 min after the onset of ischemia (treatment).

III:

The effect of levosimendan on myocardial ischemic metabolism in the presence of beta-1 adrenergic receptor antagonism was studied. Microdialysis catheters were placed in the ischemic myocardium and in non-ischemic control myocardium. A regional coronary artery was occluded for 30 min. After reperfusion the observation continued for 90 min. The animals were divided into three groups, one control group, one group where metoprolol was injected 30 min before coronary artery occlusion, and one group where levosimendan was started 30 min before coronary artery occlusion in addition to a metoprolol injection.

IV:

The course of myocardial microdialysate glycerol and glycolytic metabolites was investigated during cold cardioplegic storage. Two microdialysis catheters were placed in the myocardial interventricular septum. Microdialysate was recovered from the beating heart and hourly during ten hours of storage. Heart harvest was performed after arrest with cold crystalloid cardioplegia and stored in the cold solution. The relationship between myocardial glycerol accumulation and duration of storage was analysed.

Ethics

Animal use was approved by the Animal Research Ethical Committee in Lund. The animals were treated according to the "Guide for the Care and Use of Laboratory Animals", National Institutes of Health. General anaesthesia was used during the experiments until euthanasia by induced ventricular fibrillation (I-III) or the heart harvest procedure (IV).

Material

Directly bred domestic pigs.
I: 60-65 kg, n=6.
II: 61-82 kg, n=12.
III: 60-70 kg, n=18.
IV: 60-65 kg, n=6.

Anaesthesia

I: Induction: ketamine 1000 mg i.m., thiopental 250 mg i.v., pancuronium 8 mg i.v., atropine 0.5 mg i.v. Maintenance: fentanyl 3.75 µg/kg/h, pancuronium 0.15 mg/kg/h, nitrous oxide 65%.
II+III: Induction: ketamine 1000 mg i.m., xylazine 100 mg i.m., thiopental 250 mg i.v., pancuronium 8 mg i.v., atropine 0.5 mg i.v. Maintenance: fentanyl 5 µg/kg/h, pancuronium 0.2 mg/kg/h, nitrous oxide 65%.
IV: Induction: ketamine 1500 mg i.m., thiopental 250 mg i.v., pancuronium 8 mg i.v., atropine 0.5 mg. Maintenance: ketamine 24 mg/kg/h, midazolam 0.09 mg/kg/h, pancuronium 0.7 mg/kg/h, nitrous oxide 50%.

Ventilation

All animals were intubated through a tracheotomy and ventilated, aiming for an arterial pCO_2 of 4.5-5.5 kPa.
Used ventilators:
I+II: Servo 900.
III: Servo 900 B.
IV: Servo 300.

Fluid infusions (after early central venous access)

I: Ringer's acetate 11 ml/kg/h, Glucose 5% 1 ml/kg/h.
II: Ringer's acetate 10 ml/kg/h, Glucose 5% 1 ml/kg/h.
III: Ringer's acetate 10 ml/kg/h.
IV: Ringer's acetate 2000 ml, Dextran 70, 500 ml, and Glucose 10% 1.5 ml/kg/h (anaesthesia-carrier) during 2.5 hours before harvest procedure.

Surgical preparation

Access to the heart and great vessels was prepared through a median sternotomy.

Coronary artery preparation (I+II+III)

Preferentially the largest branch from the circumflex artery (I+II+III) or alternatively from the left anterior descending artery (I+III). A proximal snare was prepared for subsequent temporary coronary artery occlusion (CAO).

Harvest procedure (IV)

Heparin 40,000 units i.v. Cardioplegic arrest with 1000 ml cold (4°C) St. Thomas solution into the clamped aortic root. After excision, the hearts were placed in fresh cold solution and stored at 4°C.

Hemodynamic monitoring

I: MAP, CVP, HR, ST-deviation (lead II, aVL), CO, CAF, Arrhythmias.
II: MAP, CVP, PA, HR, ST-deviation, dp/dt_{max}, CO, CAF, Arrhythmias.
III: MAP, HR, dp/dt_{max}, CO, CAF, Arrhythmias.
IV: before harvest: SAP, HR.

Monitoring equipment

Pressures were monitored by intravascular catheters connected to standard pressure transducers transmitting to monitors. Arterial pressure was measured through a carotid artery, CVP in the central vena cava, and PA in the pulmonary artery. In study II+III, the transmitted arterial pressure signal was computed to derive the dp/dt_{max}. A five-electrode ECG was used for HR and ST-segment analysis. For HR, ECG, and pressures, the monitors used were the 90309 Spacelabs and the HP 78353B. In study I-III, perivascular transit-time ultrasonic probes placed at the ascending aorta and at the coronary artery that was temporarily occluded were used for measurements of CO and CAF respectively, transmitting to the Transonic HT207 monitor. Arrhythmias were registered manually.

Temperature monitoring

Body temperature was monitored in all studies by different approaches. In study IV, the temperature was monitored in the solution during storage.

Estimation of the area of ischemic myocardium (I+II+III)

I: the maximum extension of epicardial cyanosis was measured.
II+III: measured by post-mortem injection of methylthionine to delineate the "ischemic area".

Microdialysis procedure

I+II+III: Microdialysis catheters CMA 20 PC 14/10, 10 mm membrane, 20 kDa. Perfusion flow rate 2.0 µl/min, with isotonic CMA perfusion fluid T1. One catheter was placed in the "ischemic area" and one in "non-ischemic" control myocardium in the left ventricle apex. Sampling intervals: 10 min.

IV: Microdialysis catheters CMA 60, 30 mm membrane, 20 kDa. Perfusion flow rate 0.3 µl/min, with isotonic CMA perfusion fluid T1. Two catheters were placed in the interventricular myocardial septum 2-3 cm apart. Sampling intervals: one 30 min period before harvest, then 60 min periods during storage, the first sampling includes the harvest period.

The recovery after insertion before sampling was one hour in all studies.

Microdialysate analysis

I+II+III: glucose, lactate, pyruvate, glycerol.
IV: glycerol, glucose, lactate, pyruvate, urea.
By enzymatic colorimetric method with the CMA 600 Microdialysis analyzer.

Levosimendan analysis (II+III)

Plasma sampled at the end of ischemia and at the end of the experiments was sent to Orion Pharma for analysis of levosimendan concentrations.

Myoglobin analysis (III)

Plasma sampled at the end of the experiments was analyzed for concentrations of myoglobin with the Triage® Profiler S.O.B. ™ kit.

Study drugs and dosing (II+III)

II+III: Levosimendan
Levosimendan infusion solution 13.3 µg/ml.
Loading dose 13.3 µg/kg over 10 min, followed by an infusion of 0.67 µg/kg/min.
III: Metoprolol
Metoprolol tartrate solution 5 mg/ml. Injected loading dose: 0.3 mg/kg.

Experimental protocols

I: Baseline - ischemia 30 min - reperfusion 180 min.
II: Baseline - pre-ischemic period 30 min - ischemia 30 min - reperfusion 120 min.
III: Baseline - pre-ischemic period 30 min - ischemia 30 min - reperfusion 90 min.
IV: Baseline in beating heart - harvest procedure - 10 hours of cold heart storage.

Study groups

II:
PRO (n=6): levosimendan started 30 min before CAO.
TRE (n=6): levosimendan started during ischemia, 10 min after CAO.
III:
CTRL (n=6): control group.
BETA (n=6): metoprolol injected 30 min before CAO.
BETA+L (n=6): levosimendan started in addition to metoprolol dose 30 min before CAO.

Calculations

I: LPR, SV, SVR.
II: LPR, SV, SVR. ST=mean ST-segment deviation in lead II and aVL.
III: LPR.

Primary END-points

I: Course of myocardial microdialysate metabolites and hemodynamic changes during ischemia and reperfusion.
II: Between group differences for myocardial microdialysate metabolites and hemodynamic changes during ischemia and reperfusion.
III: Between group differences for myocardial microdialysate metabolites at the end of the ischemia and at the initial reperfusion.
IV: The course of myocardial microdialysate glycerol during storage.

Statistical analysis for primary end-points

I: One-way repeated measures ANOVA with Bonferroni multiple comparisons.
II: Two-way repeated measures ANOVA with Bonferroni multiple comparisons.
III: Kruskal-Wallis one-way ANOVA on ranks with Dunn's multiple comparison procedure.
IV: Friedman repeated measures ANOVA on ranks with Dunn's multiple comparison procedure. Pearson product moment correlation for glycerol concentration versus duration of storage.
Statistical software used: Sigmastat 2.03.

Study I-III, microdialysis setup.

Figure: Study I-III, microdialysis setup.
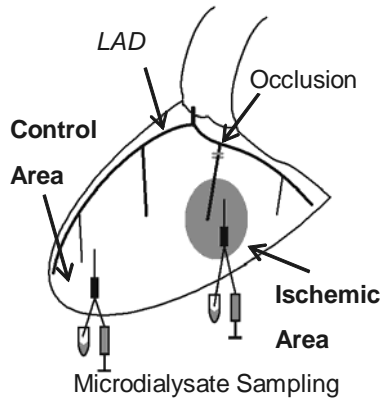Microdialysate sampling from "ischemic" and "non-ischemic" myocardium before, during, and after temporary coronary artery occlusion.

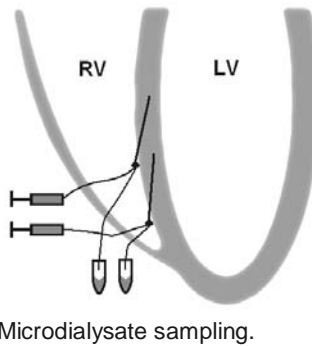## Study IV, microdialysis setup.

Figure: Study IV, microdialysis setup.
Microdialysate sampling from the myocardial interventricular septum in the beating heart and during ten hours of cold cardioplegic storage after induced arrest and harvest.

Specific microdialysis methodological issues

The CMA20 microdialysis catheters contained glycerol, a residue from the manufacturing and storage process. To washout the glycerol before use, the manufacturer's instructions were followed in study I. It was recognized that this was not fully adequate. To eliminate the glycerol before use, a laboratory procedure was developed and test performed to confirm washout. After washout, the glycerol release from the catheters (Study II+III) was below the detection limit for the glycerol concentration analysis method used in the studies, which was several times below the baseline levels of glycerol in the experiments.

In study IV, microdialysate urea was analyzed for the purpose of demonstrating any washout effect with microdialysate from the "isolated" myocardium during cold ischemia. The urea concentrations were stable during storage indicating no considerable washout.

How the cooling of the myocardium in study IV would affect the performance of the CMA60 microdialysis catheters was not known. In-vitro recovery was tested in the laboratory in a standard solution and did not change during cooling of the solution from 40°C to 4°C and a period at that temperature.

Results

I. Myocardial ischemia-reperfusion and microdialysis.

Ischemia and reperfusion Fig I-A.
Observed epicardial cyanosis during ischemia was 23 cm^2 (20-30); mean (range). There was effectively temporarily ceased flow in the occluded artery in all experiments. During ischemia, significant ischemic ST-changes were observed. At reperfusion, coronary artery flow returned with significant reperfusion hyperaemia.

Arrhythmias
A short self-limiting VT in one animal during ischemia and in another at reperfusion was observed. Five animals had temporary supraventricular tachycardia initially at reperfusion. No VF occurred.

Hemodynamics Fig I-BC.
Average CO in percent of baseline: 93% (76-102) during ischemia, and 79% (70-99) during the first 10 min of reperfusion; mean (range). Initially at reperfusion, there was a significant drop in CO, SV, and MAP. The mean CO during the initial 10 min of reperfusion was significantly lower than baseline. During the experiment, the heart rate increased significantly.

Microdialysate metabolites Fig I-D.
Myocardial microdialysate concentrations in percent of baseline at the end of ischemia and at reperfusion in the ischemic myocardium: glucose, 22% (8-38) and 61% (47-75); lactate, 659% (340-1217) and 578% (249-1082); pyruvate, 38% (9-96) and 102% (38-225); glycerol, 178% (126-287) and 181% (105-307); LPR, 2719% (1139-5031) and 743% (161-1346); mean (range). In the ischemic myocardium, during ischemia or initially at reperfusion, significant changes were observed for the concentrations of glucose, lactate, glycerol, and the lactate/pyruvate ratio. After the initial reperfusion, the glucose and pyruvate concentrations increased significantly during the experiment, and the glycerol concentration decreased. In the non-ischemic control myocardium significant changes during the experiment were observed for myocardial concentrations with increasing glucose, decreasing glycerol, and increasing pyruvate, which resulted in decreasing LPR.

Fig I-A.
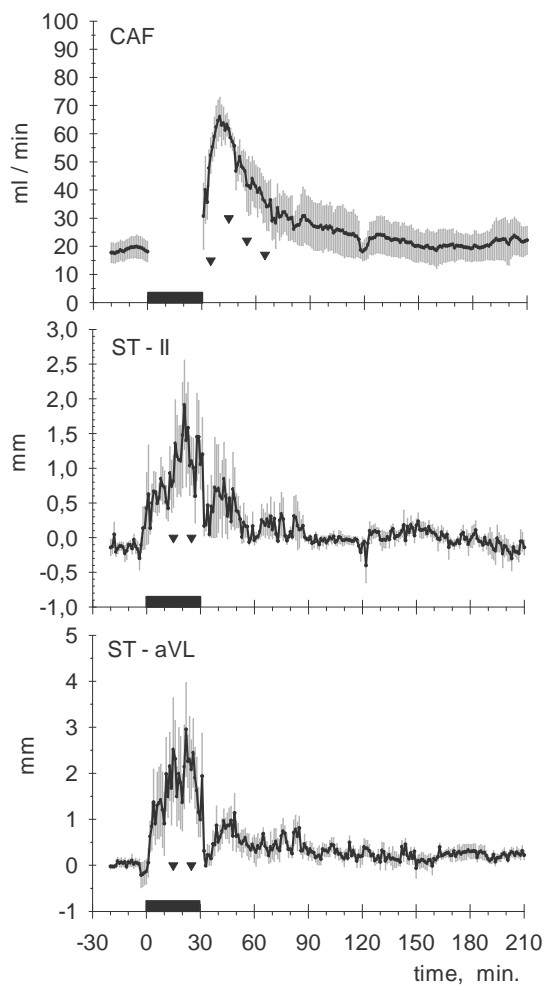
Fig I-A. Monitored hemodynamics.
Mean±SEM. CAO at time zero. Ischemic period marked by black bar. ▼, mean during the corresponding ten minute period differed significantly from baseline. ▽, significant change at reperfusion.

Fig I-B.

Fig I-B. Monitored hemodynamics.
Mean±SEM. CAO at time zero. Ischemic period marked by black bar. ▼, mean during the corresponding ten minute period differed significantly from baseline. ▽, significant change at reperfusion.

Fig I-C.

Fig I-C. Monitored hemodynamics.
Mean±SEM. CAO at time zero. Ischemic period marked by black bar. ▼, mean during the corresponding ten minute period differed significantly from baseline. ▽, significant change at reperfusion.

Fig I-D.
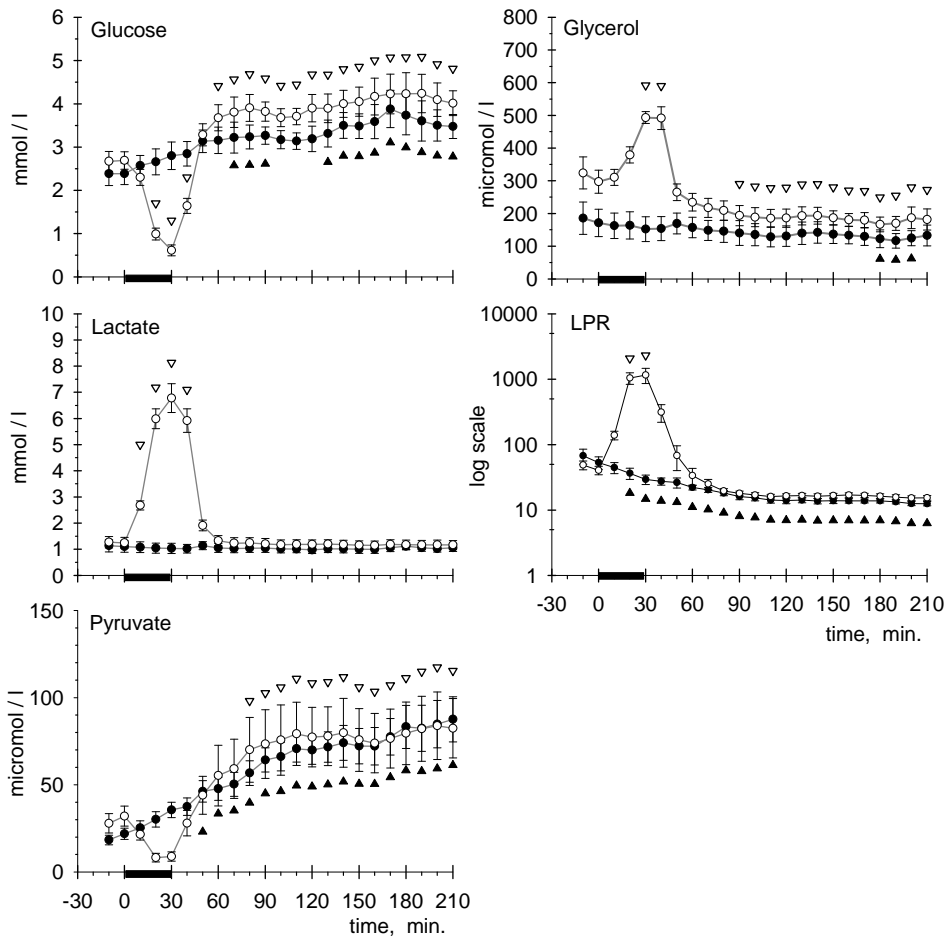
Fig I-D. Course of myocardial metabolites.
Mean±SEM. CAO at time zero. Ischemic period marked by black bar.
●, control myocardium; O, ischemic myocardium. Values differing significantly
from baseline are marked: ▲, control myocardium; ▽, ischemic myocardium.

II. Levosimendan protection versus treatment.

Ischemia and reperfusion Fig II-A.
During ischemia there was effectively occluded flow in all experiments and visible cyanosis. During ischemia and reperfusion, significant ST-segment elevations persisting to 20 min of reperfusion were observed. Significant reperfusion hyperaemia in both groups was observed. Estimation of the ischemic area: PRO=14 cm^2 (12-16), TRE=14 cm^2 (12-20); median (range).

Arrhythmias
Number of animals with VF or VT occurring during ischemia: PRO=0, TRE=2; and at reperfusion: PRO=3, TRE=3.

Hemodynamics Fig II-A.
Significant differences between the groups were found for CO, dp/dt$_{max}$, and SVR before or during ischemia. No differences were observed at reperfusion. CVP and PA were stable, without differences between groups, and are not presented in the figure.

Microdialysate metabolites Fig II-B.
In the ischemic myocardium, at the end of the ischemia and initially at reperfusion, significant differences between the groups were found for myocardial concentrations of glucose, lactate, and LPR. No differences were observed for the non-ischemic myocardium.

Levosimendan plasma concentrations
At the end of the ischemia: PRO=171 ng/ml (140-191), TRE=112 ng/ml (84-132). At the end of the experiment: PRO=202 (171-244), TRE=184 (142-210); median (range). At the end of the ischemic period the concentrations differed significantly between the groups.
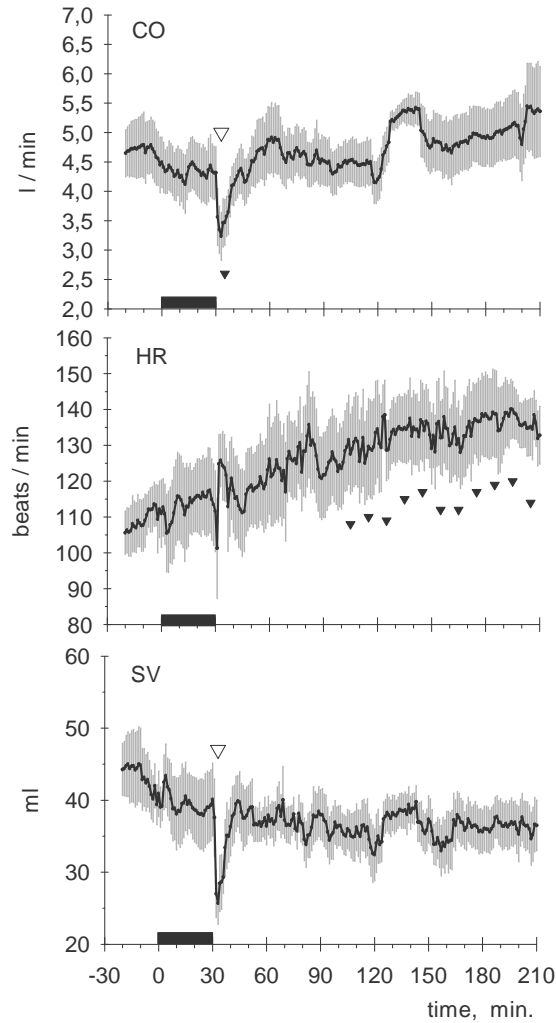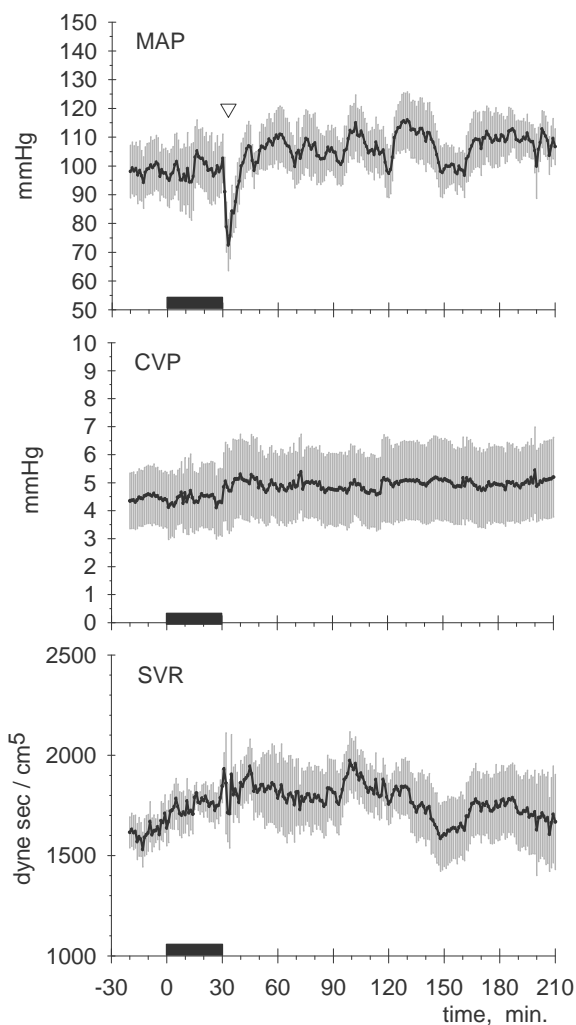
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Fig II-A. Monitored hemodynamics.
Mean±SEM. CAO at time zero. Ischemic period marked by black bar.
●, PRO; ○, TRE. +, significant difference between PRO and TRE.

Fig II-A.
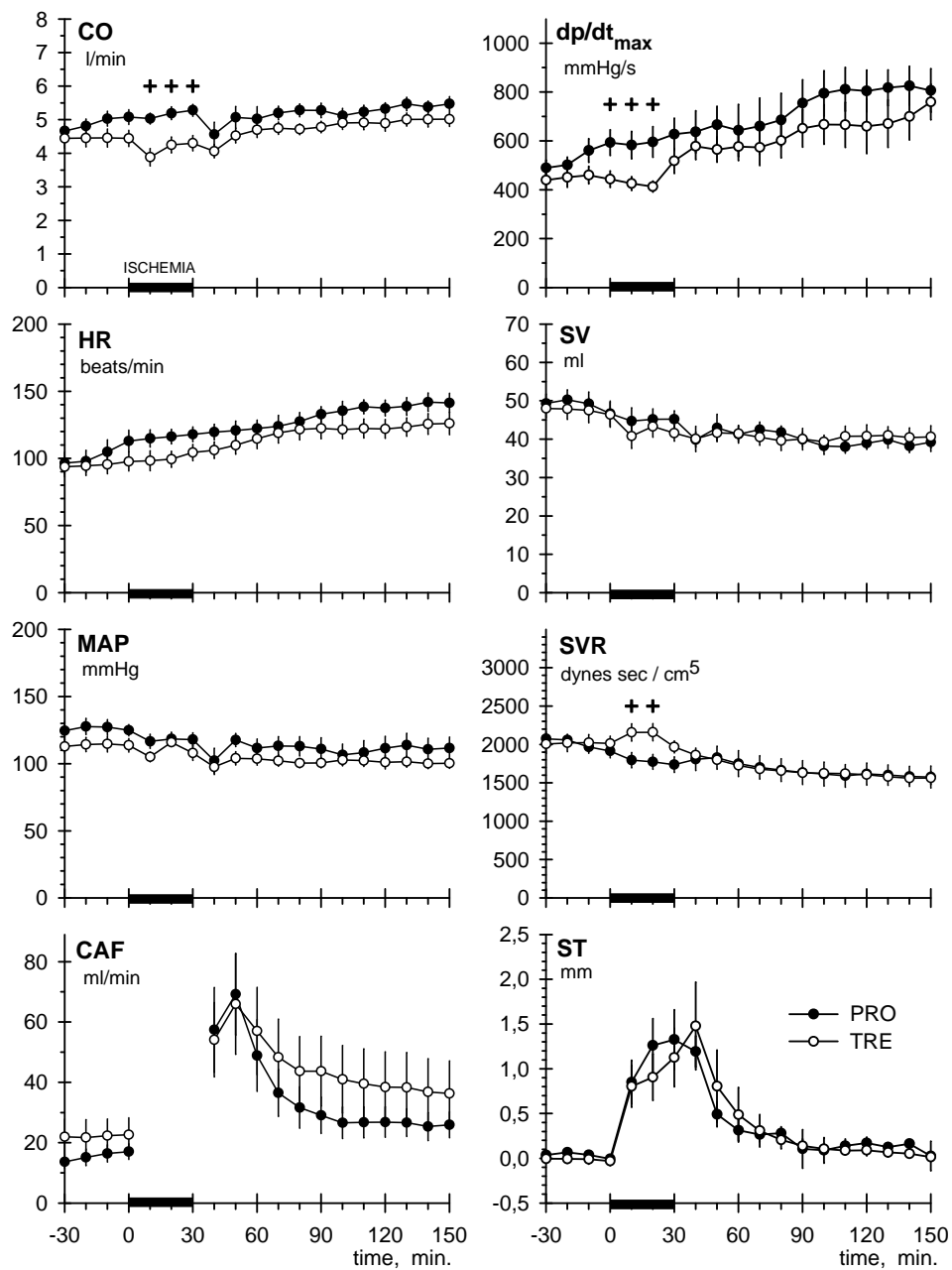
Fig II-B.

Fig II-B. Course of myocardial metabolites.
Mean±SEM. CAO at time zero. Ischemic period marked by black bar.
●, PRO non-ischemic myocardium; ▼, PRO ischemic myocardium.
○, TRE non-ischemic myocardium; ▽, TRE ischemic myocardium.
+, significant difference between PRO and TRE for ischemic myocardium.

III. Metoprolol and the potential inhibition of levosimendan protection.

Ischemia and reperfusion
During ischemia, the coronary artery flow was effectively occluded in all experiments, and cyanosis was observed. In all experiments at reperfusion, coronary artery flow increased at least 50% beyond baseline values. However, this was tested significant only for groups BETA and BETA+L. Estimation of ischemic areas: CTRL=18 cm^2 (13-48), BETA=18 cm^2 (8-25), BETA+L=19 cm^2 (12-24); median (range).

Arrhythmias
No VF occurred during ischemia. Number of animals with VF at reperfusion: CTRL=4; BETA=2; BETA+L=2.

Hemodynamics Table III.
No significant differences between groups were found. The within groups differences from baseline are presented in the table.

Microdialysate metabolites Fig III.
Significant differences were only found between the groups CTRL and BETA+L for the myocardial concentrations of glucose, glycerol, and LPR at the end of the ischemic period in the ischemic myocardium. No differences were observed at reperfusion or in the non-ischemic myocardium.

Levosimendan plasma concentrations
Group BETA+L: at the end of ischemia 151 ng/ml (114-175), and at the end of the experiment 183 ng/ml (128-251); median (range).

Plasma myoglobin concentrations
At the end of the experiment: CTRL=95 ng/ml (36-138); BETA=87 ng/ml (33-381); BETA+L=79 ng/ml (31-156); median (range); no differences.

Table III

Table 1.	BASE	Pre-Isch	End-Isch	Rep 10	Rep 20	Rep 90
CAF	ml/min					
CTRL	16.3 ± 12.5	18 ± 17.7	no flow	53.3 ± 47.4	65.2 ± 39.0	28.1 ± 26.0
BETA	10.5 ± 3.0	9.1 ± 5.9	no flow	20.6 ± 13.1	35.4 ± 19.2 §	19.1 ± 11.5
BETA+L	10 ± 6.8	9.9 ± 4.6	no flow	33.8 ± 14.0 §	35.8 ± 13.8 §	13.2 ± 7.3
CO	l/min					
CTRL	4.0 ± 1.2	4.0 ± 1.5	3.9 ± 1.2	3.2 ± 0.6 §	3.9 ± 1.1	4.1 ± 1.5
BETA	4.0 ± 0.6	3.7 ± 0.5	3.7 ± 0.6	3.5 ± 0.7 §	3.9 ± 1.2	4.3 ± 1.2
BETA+L	4.6 ± 1.1	4.6 ± 0.9	4.5 ± 1.0	3.9 ± 1.0 §	4.4 ± 0.9	4.5 ± 0.9
HR	/min					
CTRL	73 ± 13	74 ± 18	78 ± 18	103 ± 16 §	84 ± 13	100 ± 22 §
BETA	91 ± 14	84 ± 11	81 ± 11	99 ± 16	102 ± 17	104 ± 26
BETA+L	83 ± 12	89 ± 13	97 ± 19	114 ± 14 §	105 ± 18 §	125 ± 24 §
MAP	mmHg					
CTRL	103 ± 22	103 ± 23	102 ± 22	101 ± 14	112 ± 25	98 ± 27
BETA	109 ± 8	108 ± 12	108 ± 13	103 ± 5	113 ± 10	115 ± 11
BETA+L	106 ± 18	105 ± 19	98 ± 18	101 ± 11	107 ± 23	88 ± 17 §
dp/dt_{max}	mmHg/s					
CTRL	892 ± 368	965 ± 368	880 ± 328	770 ± 229	792 ± 322	879 ± 387
BETA	647 ± 250	628 ± 247	638 ± 251	686 ± 247	644 ± 253	811 ± 427
BETA+L	701 ± 134	714 ± 93	712 ± 128	680 ± 110	643 ± 154	811 ± 114

Table III. Monitored hemodynamics.
Mean±SD. §: significant difference within group compared to baseline.
BASE: baseline
Pre-Isch: last ten min before coronary artery occlusion
End-Isch: last ten min of ischemia
Rep 10:   first ten min of reperfusion
Rep 20:   second ten min of reperfusion
Rep 90:   last ten min of reperfusion

Fig III
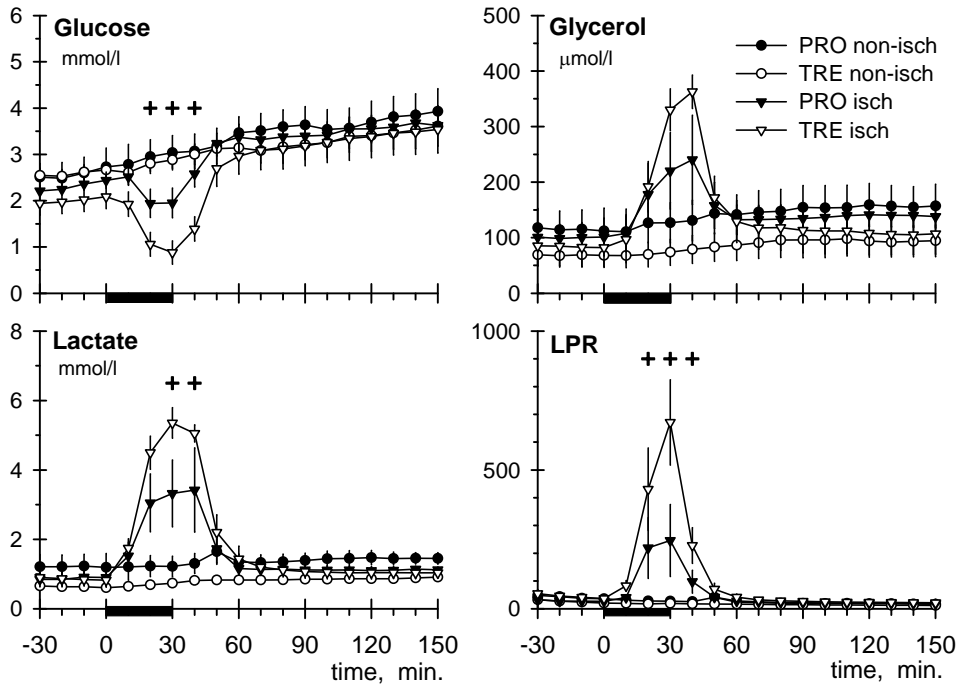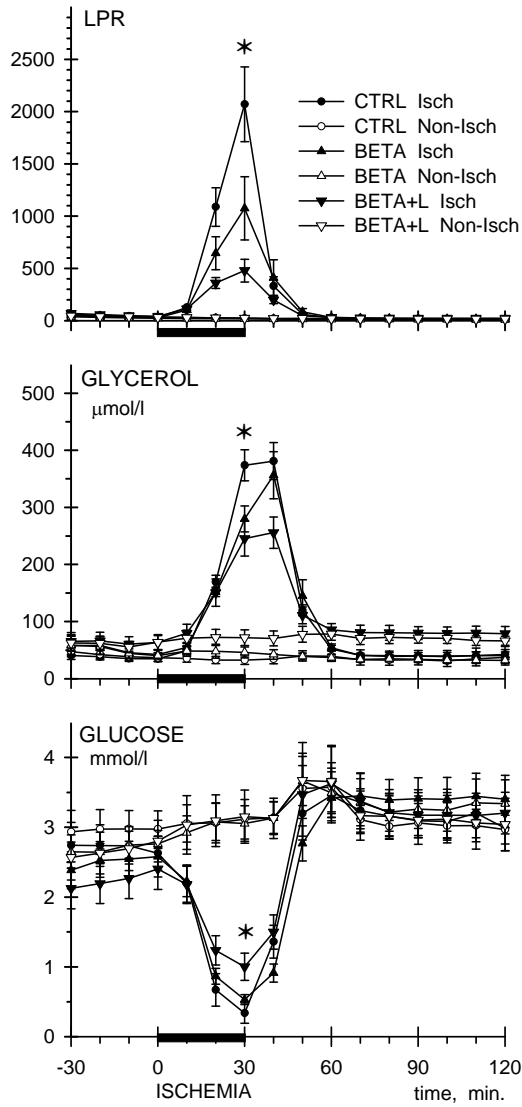

Fig III. Course of myocardial metabolites.
Mean±SEM. CAO at time zero. Ischemic period marked by black bar.
●, CTRL ischemic myocardium; O, CTRL non-ischemic myocardium.
▲, BETA ischemic myocardium; △, BETA non-ischemic myocardium.
▼, BETA+L ischemic myocardium; ▽, BETA+L non-ischemic myocardium.
∗, significant difference between CTRL and BETA+L for ischemic myocardium.

IV. Cold cardioplegic storage and glycerol accumulation.

Harvest procedure and cold storage.
The harvest procedure was done within 3 to 8 min from cardioplegia.
The temperature in the fluid during storage was 4.7 ± 1.3°C (Mean±SD).

Hemodynamics
Arterial pressure and heart rate were stable during preparation and baseline sampling before the harvest procedure.

Microdialysate metabolites Fig IV.
Ten of twelve microdialysis probes were successfully placed and functioning during the entire experiment. During cold storage 8% of glucose analyses and 66% of pyruvate analyses fell below the detection limit.

Compared to the myocardial concentrations in the beating heart before the harvest procedure (Pre-Arrest) there were significant changes during the storage period with increasing concentrations of glycerol and decreasing concentrations of glucose, pyruvate, and urea. When the course of the metabolites was compared to the myocardial concentrations at 2 hours of storage (2h Cold) there were significant changes for the concentrations of glycerol that increased and for glucose that decreased. No changes were observed for the myocardial microdialysate concentrations of lactate.

The myocardial glycerol concentrations increased during storage, and the concentrations correlated to the duration of storage. When the storage period was divided in two equal parts, an early (2-6h) and a late period (6-10h), a time-dependent correlation for glycerol concentrations was only found for the late period.
[All values presented at one hour of storage represent a mix of microdialysate obtained from before harvest procedure up to one hour of storage and were excluded from statistical analysis].

_____

Fig IV. Course of myocardial metabolites during 10 h of cold storage.
Mean±SEM. Values from beating heart (Pre-Arrest) at time zero. The course of the metabolites during storage were statistically tested against the concentrations in the beating heart (Pre-Arrest), and against the concentrations at two hours of storage (2h Cold), and the results are presented in the figure. Furthermore, the mean concentrations at 2 hours of storage (2h Cold) and at the end of the storage period (END) in percent of the Pre-Arrest concentrations are presented.

Fig IV

GLYCEROL

Percent of Pre-Arrest:
2h Cold: 95%
END: 223%

FRM ANOVA
vs Pre-Arrest : p<0.001
vs 2h Cold :   p=0.001

GLUCOSE

Percent of Pre-Arrest:
2h Cold: 23%
END: 7%

FRM ANOVA
vs Pre-Arrest : p<0.001
vs 2h Cold :   p<0.001

LACTATE

Percent of Pre-Arrest:
2h Cold: 91%
END: 87%

FRM ANOVA
vs Pre-Arrest : p=0.980
vs 2h Cold :   p=0.930

PYRUVATE

Percent of Pre-Arrest:
2h Cold: 19%
END: 18%

FRM ANOVA
vs Pre-Arrest : p=0.004
vs 2h Cold :   p=0.308

UREA

Percent of Pre-Arrest:
2h Cold: 68%
END: 64%

FRM ANOVA
vs Pre-Arrest : p=0.004
vs 2h Cold :   p=0.689

Time zero= Pre-Arrest

time, h.

DISCUSSION

In the following are discussed details relating to the model, the interpretation of the findings from the studies, some clinical implications, and the potential of microdialysis in the clinical setting related to these issues.

Anaesthesia

In studies I-III, it was intended to avoid volatile anaesthetics and propofol as both may have some cardioprotective properties (Kato 2002). Although ketamine may inhibit ischemic preconditioning (Molojavyi 2001), through inhibition of KATP channels, it was used for practical reasons for early anaesthesia induction, but not for maintenance. Pancuronium, used for muscle relaxation, may affect cardiac physiology or metabolism through a vagolytic effect. In combination with relatively high doses of fentanyl, which was not used in studies I-III, pancuronium may have a negative effect on myocardial lactate extraction (Gilbert 1990). The opioid fentanyl may have cardioprotective properties (Kato 2002), but probably in higher doses than were used in the studies I-III. Within the studies, the dosing regime was identical in all groups.

Comments on the model used in studies I-III

In study I, the local myocardial metabolism was investigated in temporary ischemic myocardium and in non-ischemic control myocardium, along with the monitoring of global circulation and regional coronary artery flow. All animals survived to the end of the experiments without observed circulatory failure. The significant and reproducible changes in some of the variables, and the relative changes compared to baseline make them eligible to investigation of the effects of anti-ischemic cardioprotection. Especially, the myocardial LPR, which in all experiments increased more than a factor 10, the interstitial glucose and glycerol, the glycerol increase at reperfusion, the cardiac output changes at reperfusion, and perhaps the hyperaemic reactive coronary flow during reperfusion were considered useful. However, not all of these variables were affected by the cardioprotective measures investigated in the following studies. The expectations of more consistent ventricular arrhythmias during ischemia and reperfusion as a potential target for cardioprotection were not fulfilled in the model.

The observed trend for the myocardial pyruvate increase in non-ischemic tissue (discussed later), the increasing myocardial glucose concentrations in non-ischemic control myocardium during the experiments, and the increasing heart rate may indicate a neuroendocrine stress-response. The inadvertent simulation of a perioperative situation was probably unavoidable because of the open chest surgical situation, but the model may be more realistic as a consequence.

Study drugs

Levosimendan
In studies II+III, the loading dose and the infusion dose of levosimendan are equivalent to 80 μg/kg/h and 40 μg/kg/h respectively. This has demonstrated similar effects on hemodynamics after comparable duration of infusion in pigs as in studies II+III (Leather 2003). The concentrations achieved in studies II+III were slightly higher.
Metoprolol
The chosen dose of metoprolol in study III was based on literature and considered to be modest. A dose of approximately 0.25 mg/kg in pigs has demonstrated an anti-ischemic effect (Ibanez 2007).

Ischemia and reperfusion

In studies I-III, the myocardium was rendered regionally ischemic, and the subsequent reperfusion was successful in all experiments. The microdialysis catheters for the detection of the metabolic changes in the ischemic myocardium were all placed centrally in the ischemic area with good margins around. Post-mortem dissection and inspection confirmed mid-myocardial positions of the catheters.

In the studies I-III, the extension of the myocardial ischemia was estimated by observation of epicardial cyanosis and post-mortem intracoronary injection of dye to delineate the "ischemic area". Throughout studies I-III, there seem to be wide ranges for the estimated ischemic areas, however no differences between groups were observed within the studies. The choice to occlude branches rather than major coronary arteries probably increased the risk of wider variations of ischemic areas. This choice was done to avoid severe circulatory failure that itself may adversely affect the metabolic variables.

In study III, in an attempt to grade the ischemic damage, myoglobin concentrations were evaluated at the end of the experiment. Wide ranges were found, with no difference between groups, and no correlation to the estimated ischemic area. It is possible that the surgical trauma itself may have contributed to the release of myoglobin from skeletal muscles.

To get further general information on the amount of ischemic myocardium in the model, one pig was used in a pilot experiment. The largest marginal branch of the circumflex artery was occluded for 30 min. With the artery occluded, blue dye was then injected into the left atrium to identify the perfused myocardium. When the staining was distributed to the myocardium, VF was induced. The heart was excised and sliced in five equally thick slices and the "ischemic area" was measured in each of these slices. The outlined "ischemic area" was longitudinal comprising the total length of the left ventricle, but only 10-15% of the circumference.

The variations observed in the studies are considered to be unimportant because the primary end-points in the studies were the metabolism in the ischemic areas during

regional ischemia without severe heart failure, and the model was successful in accomplishing that.

In studies I-III at reperfusion, a reactive hyperaemic return of coronary artery flow (Kloner 2001) was observed in all experiments with at least 50% increased flow compared to baseline at some point during reperfusion. Within the studies, there were no differences found between the groups for this variable.

Arrhythmias

In study I, only two short episodes of VT and no VF were observed during ischemia and reperfusion. However, reperfusion induced a short period with supraventricular tachycardia in most experiments. When studies II+III were included, the incidence of VT and VF was significantly higher at reperfusion compared to during ischemia. The instability of heart rhythm at reperfusion most likely contributed to the changes observed for circulation. No significant differences for the incidence of arrhythmias between the groups within the studies were observed. No significant differences for the incidence of arrhythmias between groups with or without levosimendan across the studies were found.

Hemodynamics during ischemia-reperfusion

In study I, CO, SV, and MAP temporarily decreased significantly initially at reperfusion in contrast to the less pronounced changes during ischemia. The decrease may be caused by the reperfusion induced arrhythmias and transient stunning (Ferrari 1996, Kloner 1991). However, only one short ventricular tachyarrhythmia occurred in study I at reperfusion. Furthermore, myocardial ischemia and reperfusion in one region may affect the function of remote non-ischemic regions (Marsch 1996, Marsch 1999). A decreased CO at reperfusion was also observed in all groups in study III. In none of the studies I-III did the ischemia induce a significant decrease in CO. Therefore, hemodynamic failure cannot explain the changes for the metabolic variables during this period.

Myocardial glycolytic metabolites

In study I during ischemia, myocardial microdialysate glucose, lactate, and LPR showed the expected course with signs of anaerobic glycolysis and normalisation at reperfusion. At the end of the 30 min ischemic period, the myocardial LPR was more than ten times higher than baseline in all experiments. As stated in the Introduction, an increased LPR is a good marker of myocardial ischemia. Because of different protocols, setup, and equipment used in different studies with LPR analysis during myocardial ischemia, absolute levels are difficult to compare. After 40 min of ischemia, a myocardial LPR of approximately 700 has been found in pigs (Bäckström 2004). In study I, the mean LPR after 30 min of ischemia was found to be about 1000.

The trend of increasing pyruvate concentration in the non-ischemic myocardium, in studies I-III, has also been observed in other studies with microdialysis ([1]Wikström 1995, [2]Wikström 1995, Bäckström 2004). When using a peptide stimulating insulin secretion, the pyruvate increase in the non-ischemic myocardium was inhibited ([3]Kavianipour 2003), suggesting that it was caused by a neuroendocrine response. The phenomenon of increasing pyruvate in the non-ischemic myocardium may somehow affect the use of LPR as a marker of ischemic changes, and make comparisons between different studies difficult. Within the performed studies, there were found no differences between the study groups for baseline concentrations of pyruvate.

In study IV, the continuously decreasing myocardial glucose concentrations during storage indicate that the tissue is metabolically active. However, the microdialysate lactate concentrations did not change during the ten hours of cold storage. It is possible that the produced lactate is accumulated intracellularly and not recovered by microdialysis, or that the metabolized glucose is diverted to other metabolites. Certain proteins, monocarboxylate transporters, active in lactate transport, exist in the cell membrane (Halestrap 1997). Hypothermia, at 25°C, has been demonstrated to negatively affect the efficacy of the rat skeletal muscle lactate transporters (Roth 1990). In a study of porcine cold hepatic preservation, stable microdialysate lactate concentrations have been demonstrated for up to 15 hours of storage (Nowak 2002). In contrast, when tissue lactate concentrations were analyzed in sequential biopsies from dog hearts during 24 hours of hypothermic storage, continuously increasing levels were demonstrated (Takami 1988). These findings may indicate that lactate is accumulated intracellularly. Another metabolic fate of glucose during myocardial ischemia may be to the accumulation of glucose-6-phosphate and glycerol-3-phosphate (Jeffrey 1992). The G-3-P may further be hydrolyzed and recovered as glycerol (de Groot 1994). The decrease of pyruvate to undetectable levels during hours of cold storage, observed in study IV, has also been demonstrated for pig livers (Nowak 2002). The findings on lactate and pyruvate in study IV indicate that the microdialysate lactate/pyruvate ratio is not a good marker of ischemia during the prevailing circumstances during cold cardioplegic storage. This is supported by two other microdialysis studies of porcine cold hepatic preservation, where the lactate/pyruvate ratio did not perform well as a marker during prolonged storage (Nowak 2002, Puhl 2006).

Myocardial glycerol

Glycerol accumulates in the ischemic myocardium (van Bilsen 1989, Ye 1996, Bäckström 2004). In the neurosurgical specialty, brain tissue glycerol increase is considered a marker of ischemic injury (Frykholm 2001), and high glycerol concentrations may correlate to poor clinical outcome (Peerdeman 2003).

Glycerol is integrated in triacylglycerols, which may be stored within and outside cardiomyocytes, and in phospholipids, one of the main components in cell

membranes. At least three pathways contributing to the glycerol release from cardiomyocytes during myocardial ischemia and reperfusion have been described. Triacylglycerols may be degraded by lipolysis, glycolytically produced glycerol-3-phosphate may be hydrolyzed enhanced by acidosis, and membrane phospholipids may be degraded.

Beta-oxidation of fatty acids is inhibited during ischemia, and incomplete lipid metabolism may lead to the accumulation of fatty acid intermediates (Ford 2002, Moore 1980). In myocardial ischemia, accumulation of glycerol precedes the rise in fatty acid intermediates, suggesting cycling of triacylglycerols (van Bilsen 1989). During ischemia, G-3-P produced by glycolysis may be directed to the esterification of fatty acids into triacylglycerols or hydrolyzed to glycerol (de Groot 1994). Ischemic induced activity of phospholipases may degrade phospholipids (Ford 2002), with theoretically glycerol release. The cycling of triacylglycerols and reesterification of fatty acids is energy demanding and may be a "futile cycle" aggravating energy loss during ischemia (van Bilsen 1989). Alternatively, the reesterification may be interpreted as a protective mechanism reducing the accumulation of fatty acids and lipolytic intermediates (Trach 1986). Accumulation of fatty acid intermediates may be associated with cell injury (Moore 1980, van Bilsen 1989). At reperfusion, phospholipids are probably the main source of the accumulated fatty acid intermediates (van Bilsen 1989). The accumulation of fatty acids at reperfusion, as arachidonic acid from phospholipid degradation, correlates inversely to hemodynamic recovery (de Groot 1993); this correlation was not found with the accumulation during ischemia. The lipid alterations during reperfusion seem to be different from the ischemic metabolism. No matter which of the above described pathways that contribute the most to the glycerol release during ischemia and reperfusion, the increase is signalling a process, or processes, that indicates potential or manifest injury. The relative contributions from the different pathways to the glycerol release are unknown.

The findings in study I are consistent with glycerol release as an indicator of ischemic damage. The high concentration during reperfusion despite re-established flow and decreasing LPR may represent reperfusion injury. The same pattern was demonstrated in all study groups in studies II+III as well. The high concentrations of glycerol at reperfusion were also associated in time with more pronounced circulatory changes as arrhythmias and lower cardiac output than observed in the ischemic period. Parallel or causal relationships are undefined however. In isolated rat hearts, with ischemia of different durations from 15 min to 45 min, there was a glycerol accumulation in all hearts during ischemia (de Groot 1993). However, at reperfusion after 45 min of ischemia, as compared to ischemia of shorter duration, there was a greater magnitude of glycerol release into the effluent than was expected when the accumulated concentration before reperfusion was considered (de Groot 1993). The prolonged release of glycerol after ischemia of longer duration coincided with prolonged lactate dehydrogenase release (de Groot 1993). This supports that increased severity or

duration of ischemia may lead to increased glycerol release at reperfusion indicating further injury. Again, the source of this glycerol release is not known. Glycerol as a marker of ischemic myocardial damage and possibly of reperfusion injury needs further investigation and validation.

In study IV, the myocardial microdialysate glycerol concentrations increased during the ten hours of cold cardioplegic storage without reaching a plateau. The accumulation seemed time dependent with a steeper increase at the end of the ischemic period. When the storage period was divided into an early and a late period, there was only found a correlation between glycerol concentrations and duration of storage for the late period. It is hypothesized that the first few hours of cold cardioplegic storage reflect the generally accepted donor heart ischemic time in cold storage, and that the period with accelerating glycerol concentrations may reflect progressive myocardial damage where recovery at reperfusion becomes less likely. The stability of the myocardial glycerol during the first hours of cold cardioplegic storage, in contrast to the steep glycerol increase observed during warm myocardial ischemia in study I, also supports that the myocardium was well preserved and protected during the initial storage. However, the protection has a limited duration. The absolute myocardial glycerol concentration, as well as the point in time were the accumulation accelerate, may have a potential in predicting myocardial functional recovery at donor heart reperfusion, and deserves further investigation. It is not known whether the earlier described pathways for glycerol release during myocardial ischemia also apply to prolonged cold cardioplegic storage. Studies of porcine cold hepatic preservation have demonstrated glycerol accumulation with correlation to duration of storage (Nowak 2003), and demonstrated an association between higher glycerol concentrations and other signs of tissue injury (Puhl 2006).

Metabolic anti-ischemic effects of levosimendan

Study II indicates that levosimendan is cardioprotective during myocardial ischemia-reperfusion. When levosimendan was started before ischemia (protection), as compared to start during ischemia (treatment), the myocardial ischemic metabolism showed a reduced response. The expected signs of anaerobic glycolysis were observed with increased myocardial lactate, LPR, and decreased glucose concentrations during ischemia. The changes were significantly reduced with levosimendan protection as compared to treatment, and this persisted into the initial reperfusion. A simple comparison to the data from study I supports a conclusion on protection versus the control situation as well. In the clinical setting, when myocardial ischemia is highly expected or acutely detected, either pretreatment or treatment are the alternative options. This explains the design of study II.

We cannot conclude to what extent the observed reduced response depends on induced metabolic factors or an increased coronary collateral flow. However, the

ischemia by coronary artery occlusion was complete and the metabolism was studied centrally in the ischemic myocardium.

In study II, in the protection group, the mean LPR in the ischemic myocardium at the end of 30 min of ischemia was 37% of the mean LPR in the treatment group at the same point. If compared to the mean value of LPR at the same point in study I, it was 21% of the corresponding value. In a study in pigs, with ischemic preconditioning, the mean LPR after 30 min of myocardial ischemia in the preconditioned myocardium in percent of the non-preconditioned myocardium in control animals can be estimated based on the mean results of lactate and pyruvate to be approximately 18% ([2]Wikström 1995). This may support that the cardioprotective effect of levosimendan observed in study II on the myocardial ischemic metabolism, as interpreted by the reduced myocardial LPR in percent of the control situation, is of the same magnitude as ischemic preconditioning.

The anti-ischemic effects of levosimendan have been demonstrated in other studies, as described in the Introduction. Negative study results have been presented as well (Tassani 2002). Because of different protocols and dosing, it is difficult to compare studies on protective effects of levosimendan.

Activation of KATP channels, as described in the Introduction, seems to be a plausible pathway for the cardioprotective effects of levosimendan.

Results with sevoflurane, also supposed to be a KATP channel opener, in experimental (Obal 2005) and clinical studies (De Hert 2004), indicate that better protection is probably provided when sevoflurane is present before and throughout an ischemic event as compared to presence only at reperfusion. In a study with isolated animal hearts, levosimendan was present in the perfusion fluid during different periods before or after 40 min of ischemia and 30 min of reperfusion (du Toit 2008). This was as levosimendan preconditioning with washout before ischemia, as levosimendan postconditioning at reperfusion, or as levosimendan pretreatment before ischemia without washout (du Toit 2008). All interventions reduced the infarct size compared to control, but pretreatment reduced the most (du Toit 2008). The infarct size reducing effect was lost if mitochondrial KATP channel blockers were co-administered (du Toit 2008). Furthermore, only preconditioning and pretreatment improved cardiac performance at reperfusion compared to control (du Toit 2008). In patients undergoing CABG surgery, a levosimendan loading dose before CPB reduced the postoperative troponin I release as compared to control (Tritapepe 2009). When levosimendan was initiated as an infusion without a loading dose before CPB in patients undergoing cardiac surgery, the postoperative troponin I release was not different compared to patients where the levosimendan infusion was started after removal of the aortic cross-clamp (De Hert 2008). The above results indicate that both dosing and timing may be of importance. Pretreatment before ischemia or protection continued from before and through ischemia and reperfusion may offer the best cardioprotection.

In study II, no differences for microdialysate glycerol concentrations were found during ischemia or at reperfusion. At the end of the ischemia and at reperfusion, levosimendan was present in both groups. In pigs, when levosimendan was initiated 30 min after coronary artery occlusion during 90 min of myocardial ischemia, the resulting infarct size was reduced as compared to control (Hein 2009), indicating that start during ischemia may also be of some benefit. It may be speculated, that the observed non-significant difference for myocardial glycerol between the groups in study II in the ischemic myocardium, in contrast to the significant difference for LPR, depends on glycerol being a slower marker of ischemia, and that levosimendan started early during ischemia prevented the development of a significant difference.

Levosimendan improves cardiac performance and has demonstrated anti-ischemic properties. Furthermore, levosimendan and the active metabolite OR-1896 may have a neutral effect on the myocardial oxygen consumption at concentrations inducing inotropic effects, in contrast to the increased oxygen consumption by the inotropic drug dobutamine (Banfor 2008). In cardiac surgery, where a risk of ischemic episodes as well as a risk of decreased cardiac output can be expected, patients with a low ejection fraction may have a double benefit with levosimendan. First, circulation may be optimized before deterioration occurs, and secondly, some protection may be induced against injury during ischemic episodes. Two meta-analyses from 2009 summarizing randomized controlled studies support that levosimendan in cardiac surgery patients may decrease the postoperative release of cardiac troponin ([2]Zangrillo 2009), and indicate that postoperative mortality may be decreased, at least in selected groups of patients (Landoni 2009).

Hemodynamic effects of levosimendan

Study II indicates that levosimendan may protect circulation during ischemia-reperfusion. When levosimendan was started before ischemia (protection), as compared to start during coronary occlusion (treatment), circulatory variables were better preserved. The differences in hemodynamics were caused by a combination of increased HR and contractility (dp/dt$_{max}$) with a decreased SVR resulting in preserved CO. These effects of levosimendan are known and described (Papp 2005, Toller 2006, Lehtonen 2007). However, the difference on the hemodynamic effects disappeared at reperfusion, but at that time levosimendan was present in both groups. It is interesting, that the significant within group drop in CO during the first ten minutes of reperfusion as compared to baseline that was found in study I as well as in all groups in study III, was not observed significant in any of the groups in study II. As regional ischemia and reperfusion may affect global hemodynamic performance, circulatory optimization with levosimendan before this happens may be of benefit in patients with poor ventricular function in situations were myocardial ischemia is highly expected to occur.

Levosimendan added to metoprolol

Study III indicates that the cardioprotective effect of levosimendan is not prevented by beta-1 adrenergic receptor antagonism. When levosimendan was added to metoprolol before ischemia and compared to control at the end of the ischemic period, significant lower myocardial concentrations of glycerol and LPR and higher concentrations of glucose were found in the ischemic myocardium. We cannot exclude whether the effect was attenuated by metoprolol. The results for the protection group from study II, with levosimendan and without metoprolol, at least suggest that the combination of metoprolol and levosimendan does not perform better than levosimendan alone in reducing the myocardial metabolic response to ischemia. Beta-1 adrenergic receptor antagonists, including metoprolol, have the potential to inhibit the preconditioning induced by volatile anaesthetics (Lange 2006, Lange 2008). This does not exclude other pathways to be open for signal transmission, which is supported by the demonstration that beta-1 adrenergic signalling is not essential for ischemic preconditioning (Lange 2006, Iliodromitis 2004).

As in study II, we cannot in study III differentiate between the contributions of an induced collateral flow or inducible metabolic factors on the observed protective effects. However, the pig heart, as the human heart, is supposed to be without important coronary collaterals meaning that regional ischemia by occlusion of an artery will be complete in the affected area.

In study III, the ischemic glycerol concentrations followed the same pattern as LPR and added to the conclusion of a protective effect against ischemic injury. However, no significant difference was found at reperfusion.

In study III, no differences were found between the groups on hemodynamic variables. The model used is not a heart failure model, and study III was not primarily designed to study the hemodynamic effects but merely the metabolic effects in the ischemic myocardium. As patients with a left ventricular ejection fraction of less than 30% undergoing CABG surgery and preoperatively on beta-blockers may have an increased postoperative mortality (Ferguson 2002), it may be of concern if any expected effect of therapy initiated to induce cardioprotection or to improve cardiac performance are inhibited by beta-blockers. Only few studies have described hemodynamic changes during the combination of levosimendan and beta-blockers, but not in the context of ongoing myocardial ischemia. No indications exist that beta-blockade would inhibit the circulatory effects of levosimendan. In one study, when levosimendan and carvedilol were administered to healthy subjects, the contractility response to levosimendan was not changed by the presence of carvedilol (Lehtonen 2002). In the LIDO study, it was found that in patients on beta-blocker therapy with cardiac failure, the positive effect of levosimendan on cardiac performance was not attenuated as compared to the effects of the beta-adrenergic receptor agonist dobutamine (Follath 2002). In the SURVIVE study, in a sub-group analysis, it was

found that in patients with acute heart failure requiring inotropic drugs, levosimendan decreased early mortality in patients chronically on beta-blockers as compared to dobutamine (Mebazaa 2009). In elderly patients with acute heart failure, levosimendan induced a significant improvement in cardiac index and wedge pressures in patients with and without chronic beta-blockade (Kirlidis 2009). Although not reported significant, the response in percent of baseline was only half the magnitude in patients with beta-blockers as compared to patients without (Kirlidis 2009).

Study III demonstrated that levosimendan elicited a cardioprotective effect on the myocardial ischemic metabolism in pigs beta-blocked with metoprolol. The hemodynamic effects of the combination of levosimendan and beta-blockers need further investigation.

Effects of metoprolol

In study III, no significant protective effect on metabolic variables was found with metoprolol. Anti-ischemic effects of metoprolol have previously been demonstrated as mentioned in the Introduction. The finding in study III could be explained by an insufficient number of experiments, low dose, poor timing, or that the effect in this porcine model is negligible.

In study III in the group BETA, metoprolol prevented a within group increase in HR, whereas HR increased during the experiment in the other groups. Therefore, a lowered HR in the group BETA+L does not seem to contribute to the protective effect in this group, an effect ascribed to levosimendan.

Donor heart preservation - a perspective for the future

Study IV revealed an accelerating glycerol accumulation during cold cardioplegic storage. The concentrations during the late period correlated to the duration of storage, indicating an association with progressive tissue damage. The change was diminished by the cold cardioplegia. At the end of the storage period, the myocardial glycerol concentration was 223% of baseline. This level was achieved much faster in the studies I-III during warm myocardial ischemia. With hypothermia, lowering the temperature to 25°C, the glycerol increase during ischemia in skeletal muscle was delayed (Ye 1996), demonstrating that glycerol accumulation can respond to protective measures.

However, glycerol is not (yet) validated as an injury marker quantifying myocardial damage, and it is not known whether accumulated concentrations during preservation and storage correlate to poor functional recovery at reperfusion after preservation.

It may be useful, however, if the myocardial viability can be monitored during storage, and the functional recovery predicted. This may be a prospect for future studies.

Microdialysis potential

Intravasal microdialysis in the heart, for instance in the great cardiac vein, may monitor the myocardium in a global perspective (Bäckström 2004). However, microdialysis in the myocardium directly monitors local alterations and gives specific needed information on the myocardium of investigational interest or if any changes actually occur in a specific monitored area. The monitoring of myocardial metabolism has several potential applications in experimental studies of cardioprotection or interventional effects on cardiac performance. Unfortunately, a direct access to the heart is needed. In cardiac surgery this is available and the microdialysis technique has been proven feasible (Habicht 1998), used during the surgical procedure (Bahlmann 2004), and extended into the postoperative period (Kennergren 2003), for the monitoring of myocardial ischemic changes. The early detection of deranged myocardial metabolism before the manifestation of irreversible changes may be of benefit to guide therapy. Study I supports that microdialysis may be used in the detection and investigation of effects on myocardial ischemic metabolism.

In studies of donor heart preservation, or in the evaluation of organ viability during preservation, storage, or in the future during prolonged perfusion techniques, microdialysis may have a potential. Microdialysis may probably offer a bedside potential, and may be more attractive and manageable than other methods as sequential biopsies or repeated magnetic resonance spectroscopy.

CONCLUSIONS

Regional myocardial ischemia and reperfusion induce characteristic changes in the myocardial microdialysate metabolites.

In regional myocardial ischemia, the reperfusion may induce more pronounced circulatory changes and arrhythmias than the preceding ischemia.

The high levels of myocardial glycerol initially at reperfusion, despite normalizing lactate/pyruvate ratio, indicate that glycerol release at reperfusion may be a marker of reperfusion injury.

Levosimendan started before myocardial ischemia (protection), as compared to levosimendan started during coronary artery occlusion (treatment), demonstrates a more pronounced cardioprotective effect during myocardial ischemia by reducing the response in the myocardial ischemic metabolism and by improving circulation.

The cardioprotective effect of levosimendan on myocardial ischemic metabolism with a reduction in the lactate/pyruvate ratio, less glycerol accumulation and better preserved glucose concentration during myocardial ischemia does not seem to be prevented by beta-1 adrenergic receptor antagonism with metoprolol.

During prolonged cold cardioplegic storage, myocardial glycerol concentrations demonstrate an accelerating accumulation. As the rate of accumulation is time dependent, high concentrations of glycerol may indicate progressive injury.

Whether high glycerol concentrations, or the point where the increase accelerates, correlate to reduced myocardial viability and dysfunction at reperfusion need further research.

Microdialysis may have a potential in continuous monitoring of organ preservation.

It is also concluded that the microdialysate lactate/pyruvate ratio will contribute with no useful information during prolonged cold cardioplegic storage.

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