Energy-metabolic aspects of ischemia and pre-treatment: Studies in porcine myocardium

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ABSTRACT

The focus of this thesis is to clarify mechanisms involved in protective pre-treatment of ischemia by carbon monoxide (CO) and ischemic preconditioning (IP), so that new protective therapies can be designed. This is studied in heart muscle, where the clinical gain would ultimately be to be able to prolong the period during a threatened myocardial infarction where permanent injury can be prevented. The aim is to elucidate energy metabolic relations as a basis for temporary metabolic adaptation to threatened injury in heart muscle, with focus on the biological relevance of this mechanism. All studies were conducted in an open-chest, anesthetized pig model using microdialysis sampling.

Methods: In anesthetized 40 kg pigs, regional myocardial ischemia was produced by transient snare-ligation of a branch of the left anterior descending coronary artery. Microdialysis catheters were used for local sampling of interstitial fluid in the ischemic area. In Studies 1 and 2, CO was administered before prolonged ischemia in a clinically relevant dose (5% increase in carboxyhemoglobin). In Study 2, $^{45}Ca^{2+}$ was administered locally by microperfusate to ischemic myocardium with $^{45}Ca^{2+}$ recovery used as a marker for intracellular calcium overload during ischemia. Myocardial injury markers glycerol and glutamate (and taurine in Studies 3 and 4) were measured in microdialysate. In Studies 3 and 4, IP was performed by 4 brief transient cycles of coronary occlusion and reperfusion before a prolonged index ischemic episode was performed. In Study 3, ^{14}C-marked adenosine was administered locally via microdialysis catheters in the heart muscle wall, and when this was metabolized during ischemia as an energy source, it was detected as ^{14}C-marked lactate. In Study 4, a water-soluble purine nucleoside phosphorylase inhibitor was administered to heart muscle via microdialysis which was treated by IP before an index ischemia. Markers of glycolysis were measured serially before and during ischemia for Studies 1-4. Radiolabelled markers were analyzed using liquid chromatography and scintigraphy.

Results: Study 1 results showed clear signs of metabolic advantage as far as glycolytic markers related to CO during myocardial ischemia. Study 2 results demonstrated no apparent energy metabolic advantage including for $^{45}Ca^{2+}$ recovery and no diminishment of injury markers related to the single tested carbon monoxide dose during ischemia. Study 3 showed that IP led to enhanced radio-marked adenosine consumption as an energy-metabolic substrate, and that glycolytic flow (as less glucose consumption and lactate formation) was slower in IP-treated heart muscle. Study 4 showed that local purine nucleoside phosphorylase blockade inhibits adenosine utilization as an energy-metabolic substrate during ischemia, but this did not have an effect on glycolysis or injury markers during prolonged ischemia after IP.

Conclusions: From Studies 1 and 2, we concluded that CO in this dose could show effects on glycolysis during ischemia but does not seem to confer cell protection during ischemia or early reperfusion, though CO protective effects in other doses or time frames cannot be ruled out. From Study 3 we concluded that there may be an immediate energy-metabolic explanation for why more IP-treated cells survive during prolonged ischemia. From Study 4 we concluded that experimental purine nucleoside phosphorylase blockade appears to allow interruption of IP-related adenosine utilization as an energy-metabolic substrate during prolonged ischemia without obvious effects on glycolysis, and that this requires further study to test if adenosine as an energy resource during ischemia is associated with protection during infarction.

Key words: myocardial ischemia, preconditioning, carbon monoxide, adenosine

ORIGINAL PAPERS

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

I Ahlström K, Biber B, Åberg AM, Waldenström A, Ronquist G, Abrahamsson P, Strandén P, Johansson G, Haney MF.
Metabolic responses in ischemic myocardium after inhalation of carbon monoxide.
Acta Anaesthesiologica Scandinavica 2009; 53(8): 1036-42.

II Ahlström K, Biber B, Åberg AM, Abrahamsson P, Johansson G, Ronquist G, Waldenström A, Haney MF.
Exogenous carbon monoxide does not affect cell membrane energy availability assessed by sarcolemmal calcium fluxes during myocardial ischaemia-reperfusion in the pig.
European Journal of Anaesthesiology 2011; 28(5): 356-62.

III Åberg AM, Ahlström K, Abrahamsson P, Waldenström A, Ronquist G, Hauck P, Johansson G, Biber B, Haney M.
Ischaemic pre-conditioning means an increased adenosine metabolism with decreased glycolytic flow in ischaemic pig myocardium.
Acta Anaesthesiologica Scandinavica 2010; 54(10): 1257-64.

IV Katarina Ahlström, Björn Biber, Anna-Maja Åberg, Anders Waldenström, Gunnar Ronquist, Pernilla Abrahamsson, Göran Johansson, Heléne Seeman-Lodding, Michael Haney.
Adenosine utilization as a substrate for glycolysis during myocardial ischemia after ischemic preconditioning is dependent on intact purine nucleoside phosphorylase activity.
Manuscript.

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CONTENTS

ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
CO	carbon monoxide
COHb	carboxyhemoglobin
HPLC	high performance liquid chromatography
IP	ischemic preconditioning
kD	kiloDalton
PNP	purine nucleoside phosphorylase
TCA	tricarboxylic acid cycle

INTRODUCTION

When vital organs suddenly lose their blood supply, for example when there is a sudden occlusion of a coronary artery, there is a limited time during which the heart cells can survive. When blood flow is stopped, then the heart cells find themselves in a condition where they have to survive on existing nutrients and energy stores, since for the moment, no blood flow is bringing more nutrients, like oxygen, and taking away the collecting waste products of cell metabolism (like hydrogen ion, and other substances). This condition is called ischemia, and this is the condition which I have chosen to study in my thesis.

For cells exposed to ischemic conditions, things can go either way. The cell can sustain an injury from which it cannot survive, and cell death follows. The cell can sustain a minor injury, from which it can recover. The heart as an organ is particularly vulnerable to ischemia since it is a steadily working muscle. Ischemic heart disease is one of the most common life-threatening health problems in our modern Western world. To find better treatments to help cell survival during and after ischemic injury to the heart is an area of very broad and active research.

Cells have some internal mechanisms which help them survive in the face of insult or threatened injury, such as ischemia. Some of these mechanisms involve mitigating or limiting the injury cascade (Kajimura M et al. 2010, Weitzberg E et al. 2010, Downey JM et al. 2007). Others involve influencing cell machinery so that the cell can tolerate more threat or injury without dying (Murry C et al. 1986, Tekin D et al. 2010). This thesis is focused on how myocardial cells tolerate ischemic conditions, and possible mechanisms for how some of these cell survival adaptations might work.

If we can understand the mechanisms that lead to better cell survival related to some of the different protective interventions (here carbon monoxide and ischemic preconditioning), then we might be able to find a new means to deliver these same protective effects, though not in the form of a ‘poisonous’ gas or short periods of ischemia.

Can we find new and potent ways to extent this time period where cells can survive when they are suddenly deprived of their supply of substrates, like oxygen and glucose? The clinical value of this is if new and potent means to extend this period where cells tolerate ischemia without dying can be found, then this could extend the period of time where help can be found (for instance in revascularization), or extend the period of time that organs exposed to planned ischemia can survive (for instance in the cases of planned interruption of the circulation or organ transplantation). The clinical example of this is that a better and widely available protective strategy could lead to a longer period of time that heart cells could survive and benefit from reopening of an occluded coronary vessel. In the case of transplantation, with obligatory ischemic period, a better protective strategy would help transplant organ cells be in better condition after

their ischemic period, and potentially extend the period of ischemic time during which the cells remain viable.

This is the motivation behind the studies in this thesis: to try to find new ways to pre-treat, so that vital organ tissue can survive ischemia longer. In these works, we have chosen an experimental model, the heart in a large animal model, and examined the very early energy metabolic events in ischemic heart muscle as a focused and limited part of this general question of vital organ protection. While we in general are motivated to try to find means of vital organ protection, we have focused on the energy metabolic aspect of early ischemia to see if cell energetic are involved or affected by the 2 pretreatments which we have chosen to study.

So, it is energy metabolic events during ischemia which are sampled in vivo which are the main part of this thesis, and the time aspect of the assessment is limited to acute ischemia, and in one case, immediate reperfusion. Since cell injury typically occurs in a later time frame, we must make reference to other studies which have employed similar pretreatments, and have examined a far longer period after ischemia to assess for protection in terms of cell survival. If this time is very extended, then all the cells that have lost their blood supply will succumb. If this time is very brief (seconds/minutes), then most or all of the cells will escape permanent injury. If, however, there is a moderate period of time (minutes/hours) without blood supply, and supply of nutrients to the cells, then there is cell injury, and some cells survive while others will go on (after several hours) and die.

What happens during ischemia? First, the cells deplete their readily available energy resources. Much energy goes into membrane ion pumps which are needed to maintain ion gradients across cell membranes in, for example, heart cells. Heart cells store energy as glycogen, but the largest amount energy that they consume is in aerobic metabolism, breaking down energy rich free fatty acids (60-90 % during resting conditions) and other substances such as ketones and lactate which enter the tri-carboxylic acid cycle in 2 carbon units, ultimately providing substrates for oxidative phosphorylation (requiring oxygen) and high energy phosphate bond production (typically as ATP). ATP is the energy currency for the cells, and ATP is even needed to initiate some of the energy-producing processes, including for example, generating glucose from stored glycogen. This is relevant in ischemic conditions, where ATP becomes scarce, and is pretty quickly not available to help to break down glycogen to glucose, leading to glycogen at some point within minutes no longer being able to provide glucose to feed the glycolytic pathway during ischemia (book, Katz AM, 2010).

Once oxygen is not available when cells have become ischemic, the cells switch over as long as possible to anaerobic metabolism. They still have high energy needs, but the energy has to come from some source other than oxidative phosphorylation, which is so effective at producing ATP. Glycolysis becomes more relevant as far as producing energy early on during ischemia, were a limited amount of ATP can be produced without needing oxygen, though local glucose

stores provide what they can. As mentioned above, when ATP is depleted, then glycogen can no longer be hydrolyzed to produce glucose, and this energy source is only briefly available in ischemic conditions.

Prolonged ischemia, from the cell's perspective, is essentially a rapidly developing scenario where ATP becomes scarce, and where vital cell functions that are energy-dependent slow and then stop (book, Katz AM. 2010). These functions include among other things membrane ion pumps (Murphy E et al. 2008).

Normal energy metabolism for cardiomyocytes includes consumption of glucose in glycolysis ultimately to pyruvate, and then entry in the form of 2 carbon fragments into the TCA cycle, where the largest part of the ATP is produced. Delivery of glucose locally is accomplished by capillary nutrient flow, and local coronary blood flow/delivery is in normal circumstances tightly matched to local cardiomyocyte metabolic activity, where more metabolic activity leads to reduction of local arteriolar tone and blood flow. Local glycolytic activity can be observed in many ways, and local glycolytic activity in anaerobic or ischemic conditions has been commonly reported as net lactate production in coronary sinus blood. Cardiomyocytes can consume lactate as an energy source as well, but when there is a rapid and dramatic increase in lactate levels locally, this has traditionally been associated with either an inability to receive and/or use oxygen, for example as in carbon monoxide poisoning or cyanide poisoning also affecting cytochrome c (Bauer I et al. 2009). While regional-, whole organ-, or whole body blood sampling can be used to try to identify lactate excess associated with oxygen deficit or ischemia, it is possible to place a sampling device in the interstitial space, between cardiomyocytes, in the specific part of the heart that one wants to study. We have employed microdialysis in this way.

Energy production during ischemia has been assessed in this thesis largely in terms of glycolysis, examining local glucose and pyruvate consumption in ischemic myocardium as well as production of lactate. One of the main themes in Studies 3 and 4 of the thesis is that in the right setting, a substance that becomes abundant or overabundant during ischemia may be able to enter the glycolytic pathway and produce some ATP. Adenosine becomes very abundant during ischemia, as a by-product of the accumulation of AMP. Reducing locally the amount of AMP, by metabolism of the AMP breakdown product adenosine, will allow more ADP to be hydrolyzed as an energy source.

Adenosine has long been recognized as an important receptor agonist during ischemia (Wikström G et al. 2001) and it is possible that strong adenosine receptor agonism activates some part of the metabolic mechanism that we are studying here (PNP enzyme system), though these investigations are focused at the adenosine receptor system. We have focused on the adenosine molecule itself as an energy source.

Different possible types of protection during and after ischemia

Cells own many different mechanisms for adapting to stress or threat, and it is well recognized that cells that are stressed in some way can sometimes demonstrate a transient but clearly improved survivability when a new stress or threat is present.

One early example of this is heat shock proteins (Richter K et al. 2010). Another is hypoxia inducible factor (Majmundar AJ et al. 2010). These molecules have been isolated as substances that have been elaborated within stressed or preconditioned cells. These substances when isolated seem to be able to have effects on other cells. It is not clear if this type of adaptation in general is dependent on soluble mediators or if there is just a transient change in cell function or structure that confers protection. In the case of ischemic preconditioning, we are postulating that ischemic stress leads to, among other things, up-regulation of a particular enzyme system and to the opening of a normally relatively quiet metabolic pathway.

One aspect of possible protection which receives much current attention related to ischemic preconditioning is the prevention of cascade of mediators and events in the reperfusion stage which leads to cell death, for example ultimately through mitochondrial transition pore activation. (Murphy E et al. 2008, Halestrap AP et al. 2007). Pharmacological interventions can also provide protection, (De Hert SG et al. 2009) not the least drugs used in connection with anesthesia (Frässdorf J et al. 2009).

It is possible that an energy metabolic explanation contributes to improved survival when cells are exposed to ischemia. If cells, due to an intervention, either have more energy available in the setting of energy crisis, or have lower energy needs, then this could provide the margin that is needed when a cell is balancing between survival and non-survival. It is in this perspective that we have focused on the immediate energy-metabolic conditions during ischemia in an in vivo model. These immediate conditions are not directly indicative of survival, though we are testing interventions that are known in other models to confer improved survivability during a later prolonged ischemia.

Infarction and necrosis are strongly linked to duration of ischemia. If the duration is long enough, then the cell machinery will ultimately receive a fatal injury, though it is possible that that injury occurs at different rates for different cells. One hypothesis is that a lower level of energy consumption may lead to better chances of survival or a longer interval where cell survival is possible. This may be supported by the knowledge that hypothermia is protective for vital tissue if in place before the ischemia and ischemic injury starts (Hale SL et al. 2011).

The biological role of carbon monoxide

There are several small gaseous molecules, including nitric oxide, hydrogen sulfide and carbon monoxide, which can act directly on cells organelles or as messengers (Kajimura M et al. 2010, Wagner F et al. 2009, Weitzberg E et al. 2010). These substances are produced in the body normally in small amounts though widely throughout the body, and are presumed to act locally as second messengers in vital processes. These molecules all have been identified as protective in some circumstances, though also associated with disease or injury in other circumstances.

Carbon monoxide is generated by breakdown of heme proteins, and also the heme degradation product biliverdin, by the enzyme heme-oxygenase. Carbon monoxide exists in low concentrations in the body, typically bound to hemoglobin as carboxyhemoglobin. Carbon monoxide has a very much stronger affinity than oxygen for hemoglobin. Ultimately, carbon monoxide is eliminated from the body by disassociation from hemoglobin in the lung and then exhalation. Carbon dioxide is believed to diffuse freely across cell membranes. Carbon monoxide has a strong affinity to hemoglobin as well as some other molecules such as metalloproteins, and it is this strong affinity which is associated with toxic effects when carbon monoxide is present in significant amounts in the body. How much is needed to produce symptoms must depend on the circulatory margin an individual patient has for oxygen delivery to vital organs. Exactly how carbon monoxide is protective, or if there are multiple mechanisms which can confer protection, is not clear yet.

Carbon monoxides toxic effects

Carbon monoxide poisoning is defined as an intoxication where carbon monoxide occupies binding sites including on hemoglobin, where minimum adequate oxygen transport (normally transports oxygen as oxyhemoglobin where oxygen dissociates in vital organ capillaries to diffuse to cells) is blocked leading to oxygen deprivation in vital organs (Bauer I et al. 2009). Carbon monoxide also binds to other heme molecules including cytochromes in mitochondria, which lead to interruption of oxidative phosphorylation.

Carbon monoxide protective effects

Reported carbon monoxide protective effects include in cardiomyocytes in vivo exposed to ischemia (Uemura K et al. 2005), in ischemia models for myocardial infarction (Guo Y et al. 2004) and in other ischemia models (Goebel U et al. 2008) (Goebel U et al. 2011).

Different possible mechanisms of carbon monoxide protection

Carbon monoxide, delivered early or late in the myocardial ischemia and reperfusion process has been shown to confer protection, though typically this protection is evaluated late (many hours) after CO delivery. From these findings, it is not clear if CO affects the ischemic process, the reperfusion process, or other secondary processes including, for example, local inflammation.

Carbon monoxide effects on metabolism

A common theme for the 3 gaseous second messenger molecules (Kajimur et al. 2010) is that they may induce a resting condition where injury may be better tolerated, though this theory has not been clearly proved across many species (Volpato GP et al. 2008, Baumgart K et al. 2010, Weitzber E et al. 2010). This is an effect which appears to involve energy metabolism. This is the type of effect which we have aimed to study for carbon monoxide during acute ischemia in vivo.

AIMS

Study I: To illustrate glycolysis and lactate accumulation during ischemia in heart muscle *in vivo* in relation to carbon monoxide pre-treatment and dose-finding

Study II: To assess carbon monoxide effects on metabolism and injury in ischemic heart muscle *in vivo*, including the post-ischemia immediate recovery period

Study III: To assess adenosine utilization as an energy-metabolic substrate during myocardial ischemia related to ischemic preconditioning, and with a focus on glycolysis

Study IV: To test the effect of inhibition of the enzyme purine nucleoside phosphorylase on the utilization of adenosine as an energy metabolic substrate in ischemic preconditioned myocardium

METHODS

All experiments were conducted in the large animal laboratory at Umeå University. This large animal model served as a means to test these questions in vivo in an acute injury model. To test these same hypotheses in patient material would not have been possible, since the myocardial ischemia is very difficult to arrange for a very specific heart region where one knows before-hand that one will need to sample very locally (Bäckström T et al. 2004).

The animal model

We used juvenile pigs that were around 40 kg. These have been chosen since the pig heart circulation is very similar to humans, particularly with respect to collateral vessel circulation. There is minimal collateral circulation in pigs, as in humans who have not had chronic ischemic heart disease. The heart in a 40 kg pig is large enough that it is predictably possible to isolate a branch of the left anterior descending artery which supplies a region of the left ventricular anterior wall, and then also place multiple microdialysis probes into the area that one expects to become ischemic with transient occlusion of that diagonal artery.

The pigs were of a Swedish domestic race, and were supplied by a local city public school agricultural program and they have supplied pigs to the lab for many years, where it has been noted that the animals have been consistently in good health. Animals of that age and size were not sexually mature. The animals each week were generally siblings, and were brought to the University one or two days before their experiment day. They were kept overnight in a large pen in the lab building.

The animals were first sedated with an intramuscular combination of ketamine, xylazine and atropine, so that they could be transported in a calm state to the laboratory. There they received an intravenous cannulae, and were anesthetized using a combination of barbiturates, opiates and benzodiazepine as intravenous infusions. They received no muscle relaxants. Since the experiments were acute and the animals were euthanized at the end of the experiment, a tracheostomy was performed. The animals were ventilated and provided with intravenous fluids in order to maintain them in a normoventilated, normoxic state with normal blood pressure throughout the experiments.

Preparation

The same general type of preparation was used for the animals for all four studies. Vascular access for arterial and venous cannulation was done through cut-downs onto neck vessels. Generally, the external jugular vein system was used for central venous catheterization and an external carotid artery was used for arterial cannulation.

A midline sternotomy was performed to expose the heart. The pericardium was opened, so that there was free access to the anterior wall of the heart. A larger diagonal branch of the left anterior descending coronary artery was chosen for a snare, based on the appearance that it supplied a distinct portion of the anterior left ventricle. In few case, there was not a single clear second diagonal branch, but instead there were 2 smaller branches, which lay very close to each other, supplying a clear band of anterior left ventricular wall. In that type of case, we used 2 snares in order to try to ensure that the size of the ischemic area was large enough to include the multiple microdialysis catheters that were to be placed.

We did not test the snares ahead of time to identify the ischemic area before placing the microdialysis catheters, but rather made a best guess by following the course of the diagonal artery until it penetrated the myocardium and could no longer be directly observed. This mean placing the microdialysis probes as much as possible under the diagonal branches. For me there was a learning process in this preparation, and placing the probes right where I wanted them to be in the ventricular wall of a beating heart took quite a bit of practice. This procedure was performed by selecting a location for the membrane, then measuring where the puncture point in the heart surface would need to be in order to have the membrane ideally placed. The microdialysis catheters have a hub with eyelets to allow fixation to the organ surface. A suture was placed through the eyelet, so that once we sharply placed an introducer sheath in the right location, we could put the catheter into the plastic introducer sheath and then fix the hub to the spot, allowing us to be able to split and remove the introducer without dislocating the microdialysis probe.

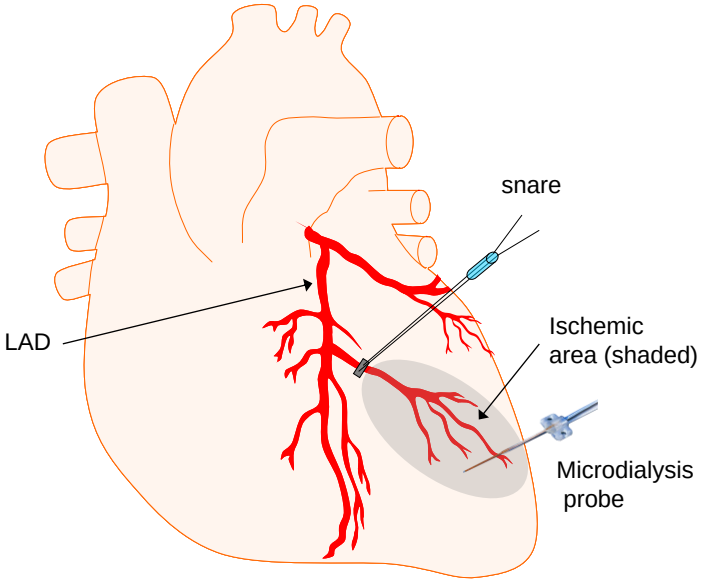

Figure 1. This schematic figure shows how a diagonal branch of the left anterior descending (LAD) coronary artery was chosen, based on an obvious relation of a branch to an area of supply on the anterior wall of the left ventricle. A snare was placed proximally around this diagonal branch. The distal aspects of the diagonal branch are intramural and not on the epicardium.

The clinical definition of ischemia is the interruption of arterial inflow to an area, and this leads to hypoxic conditions in that tissue that is dependent on that arterial inflow for its nutrients. There can be some local flow to tissue where the arterial inflow is interrupted and this local collateral flow can occur through small vessels from neighboring regions. Collateral flow and small vessel flow in general, is dependent on local vascular tone. Collateral flow may not occur in conditions where there is adequate arterial blood flow to a region. During ischemic conditions, however, where local small vessel tone is maximally reduced, some local flow can occur which may partially relieve or resupply the area. Collateral vessels are not developed in healthy pigs, though they can be developed (as in humans) through step-wise coronary occlusion (de Groot D et al. 2011).

Collateral flow, if present on the margins on the ischemic area, can make these marginal areas transiently or intermittently ischemic. This is relevant in the coronary snare model concerning very localized microdialysis sampling, since sampling from a completely ischemic area is the aim. If sampling occurs across a marginal area, where ischemia is intermittent, incomplete or absent for tissue that is immediately next to part of the microdialysis membrane, then the microdialysate result will reflect a dilution or incomplete sampling of the ischemic event. The practical implication of this is that microdialysis results for each catheter need to be judged for their adequacy of ischemia sampling before they can be interpreted as far as

ischemic changes.

Myocardial ischemia is also described in terms of an imbalance between supply of myocardial nutrients to working heart tissue and the local metabolic demand or utilization of those nutrients. The heart has a very potent mechanism for increasing local blood flow when metabolic activity or demands increase, so it is not practical to induce ischemia by simply increasing metabolic demands or activity. To experimentally bring about acute ischemia, there must be limitation in supply. If the ischemia is very brief (seconds), then the cell may not have any lasting effects. If the ischemia is longer, many seconds/minutes, then cells mechanical function is affected and impaired. If the ischemia is prolonged in a working cell, then injury can occur. Myocardial injury in the clinical setting is detected by biomarkers selected for relative specificity for heart muscle cells, including M band creatinine phosphokinase and troponins (Kelley WE et al. 2009). Prolonged ischemia leads to more injury and injury marker release, and this has even been reported with microdialysis in heart muscle (Mantovani V et al. 2002), though troponin recovery in microdialysate is poor in catheters with membrane pore sizes less than 100 kD (as we employ in our studies focused on smaller molecules, own unpublished lab results for microdialysis and troponin I). When ischemic times are approximately 45 minutes, then porcine myocardium demonstrates after some hours a significant infarction, around 50 percent of the area at risk (Näslund U et al. 1992). For longer ischemia periods, 90 minutes for example, the infarction becomes almost complete for the area at risk, at least in a healthy animal not previously exposed to ischemia or another protective pretreatment.

Microdialysis catheters allow sampling of interstitial fluid substance composition in solid organs (Abrahamsson P et al. 2011) but even also in fluids (Abrahamsson P et al. 2005). Microdialysis sampling is a means of collecting serial measurements from exactly the same local place in a solid organ, like the heart. The way that microdialysis works is that a microperfusate is delivered at a controlled rate to an area of the catheter where there is a semipermeable membrane. There is exchange of solutes across the membrane based on concentration gradients, diffusion, and limitations related to membrane pore size. The slower the flow of microperfusate, the longer the time will be for equilibration of solutes across the membrane. The better the equilibration, the better the ‘recovery’ is in the microdialysate that is collected and analysed. There is always a balance between microperfusate flow rates, which when higher allow more volume in the collection and hence a possibility for briefer sampling intervals (perhaps 5 or 10 minutes are the briefest practical sampling intervals for the types of measurements that we are performing). When the microperfusate flow rate is very high, and there is too little time for diffusion of solutes across the membrane, then ‘recovery’ is low, and the microdialysate sample concentrations are much lower than the interstitial concentrations.

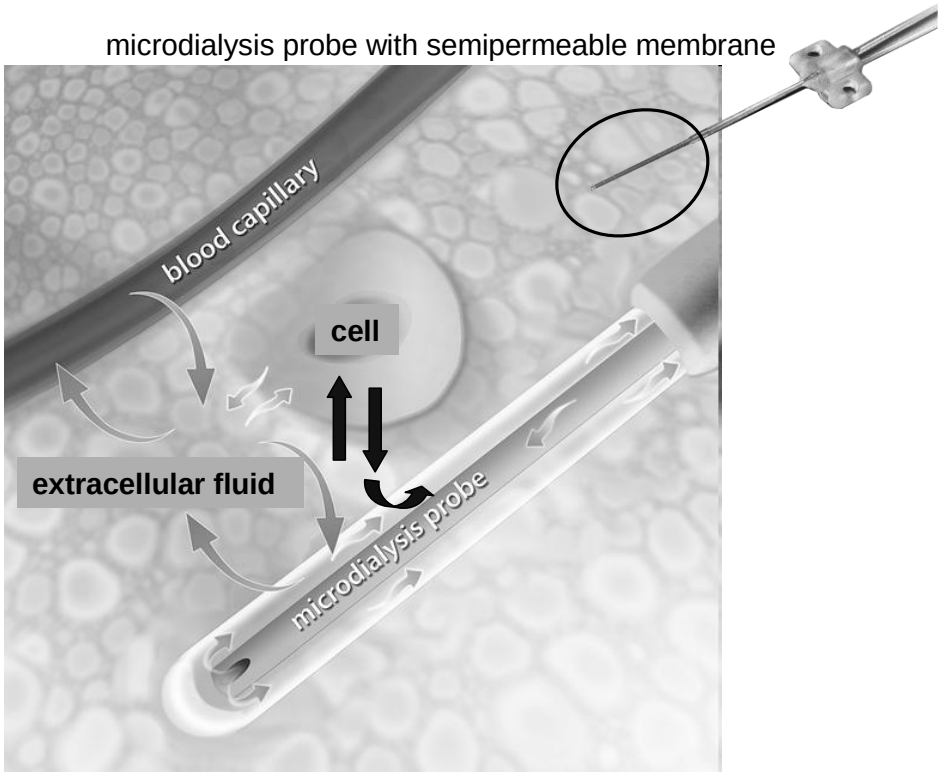

Figure 2. The microdialysis probe serves a similar function to the local capillaries. There is sampling of the substances in the interstitium, and we are focused on specific substances which are taken up by and produced by cardiomyocytes (cells). We should not forget that the capillaries also deliver and remove substances from the same area. (Background image used with permission from CMA).

The substances that are measured in microdialysate samples are chosen based on the scientific question that is being studied and the microdialysis set-up (specific catheter, membrane pore size, microdialysate flow rates) must be correct for allowing good recovery for the substances of interest. How the microdialysate samples are analysed is a separate issue. The samples must be managed optimally as far as minimizing evaporation during the storage and measurement process (Abrahamsson P et al. 2008). There are commercial systems (we have used the CMA 600 analysis machine) for reliable automateded assessments of a limited number of substances, including glucose, lactate, pyruvate, glutamate and glycerol (the cells which we operate). These are colorometric assays in the CMA 600 analysis system. These have been validated in our own lab in Umeå versus high performance liquid chromatography (HPLC) measurements of the same substances, same samples, with very good agreement (data unpublished, Pernilla Abrahamsson, Kristina Eriksson). High performance liquid chromatography has been very important in this project as a means to analyze other metabolic or injury markers including adenosine and taurine. In earlier studies in the same lab, a wide

variety of metabolic substances and amino acids have been analyzed by HPLC, though in my studies we have focused on adenosine as a key substance and taurine as an injury marker.

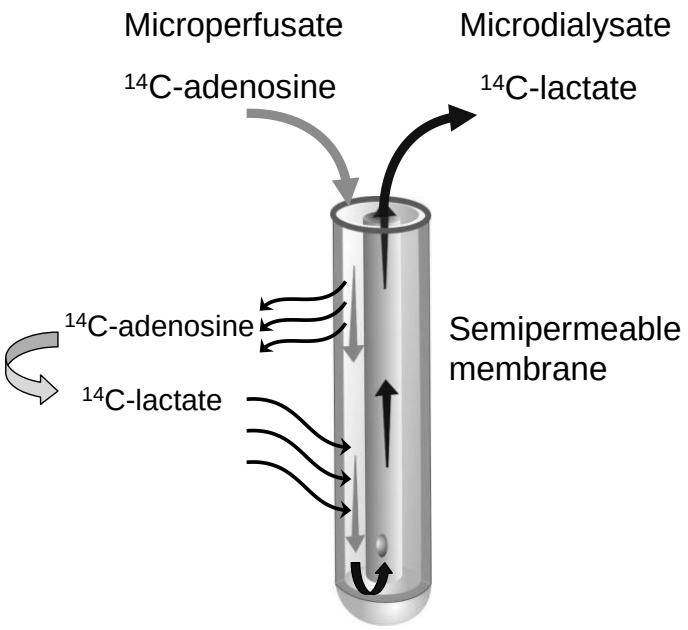

Figure 3. Microdialysis catheter showing delivery of ^{14}C-adenosine and sampling of ^{14}C-lactate. Local uptake and utilisation of ^{14}C marked adenosine produces ^{14}C marked lactate.

One of the innovative aspects in these studies is that we have used microdialysis to deliver markers to the local tissue and also collect the results. We have delivered radio-marked adenosine (^{14}C) and then collected and measured for the same ^{14}C marker as part of lactate. The ^{14}C -lactate that could be measured could only have resulted from ^{14}C-adenosine that diffused out of the microperfusate, was taken up in cells, metabolized to lactate (measured during ischemia) and then collected as ^{14}C -lactate that had diffused out of the cells and then be 'recovered' in microdialysate. This is very attractive in in vivo ischemia studies, since once there is coronary occlusion (ischemia), then there is no longer a way to deliver markers to the ischemic area through the coronary circulation.

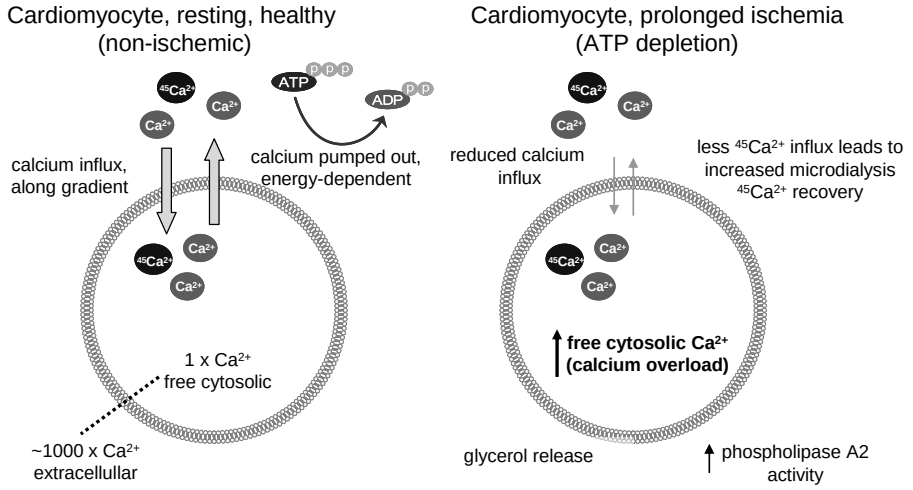

Figure 4. At rest and in health, cardiomyocytes manage calcium influx using ATP-dependent calcium pumps to pump calcium out again and avoid calcium overload. During ischemia, when ATP is no longer locally available for these ion pumps, calcium influx proceeds and calcium accumulates. This calcium accumulation during ischemia also activates phospholipase A2, which leads to liberation and release of glycerol. When the cells energy sources are replenished, then calcium is pumped out. $^{45}Ca^{2+}$ distributes itself in the calcium pool, initially extracellularly, and then also into cells (see below).

Another marker that is delivered and then sampled using microdialysis during ischemia is $^{45}Ca^{2+}$. When cells are healthy and $^{45}Ca^{2+}$ is delivered locally to the interstitial fluid via microperfusate and equilibrate across the microdialysis membrane, this calcium is to some extent taken up into cells. The amount of diffusion away from the catheter area and uptake into cells determines the amount of $^{45}Ca^{2+}$ recovery in the microdialysate. Normally, calcium ions leak into cells, since there is at rest approximately 1000 times higher calcium concentration in the interstitial fluid compared to the cytosol on the other side of the cell sarcolemma/ plasma membrane. The calcium ions are then either included in the intracellular calcium pool (pumped into the sarcoplasmic reticulum, for example) or extrude from cells. When cells become ischemic, and intracellular calcium overload begins, due to less energy available to drive the trans-membrane calcium pumps, then less calcium diffuses into cells (decreased gradient for calcium across the cell membrane). Less calcium (including $^{45}Ca^{2+}$ diffusing into cells means that there is more that remains in the interstitial fluid and more $^{45}Ca^{2+}$ recovery in the microdialysate (Engström I et al. 1995).

This microdialysis application of an earlier demonstrated principle of cell uptake of $^{45}Ca^{2+}$ is an innovation which allows assessment of degree of calcium overload in ischemic cells in vivo.

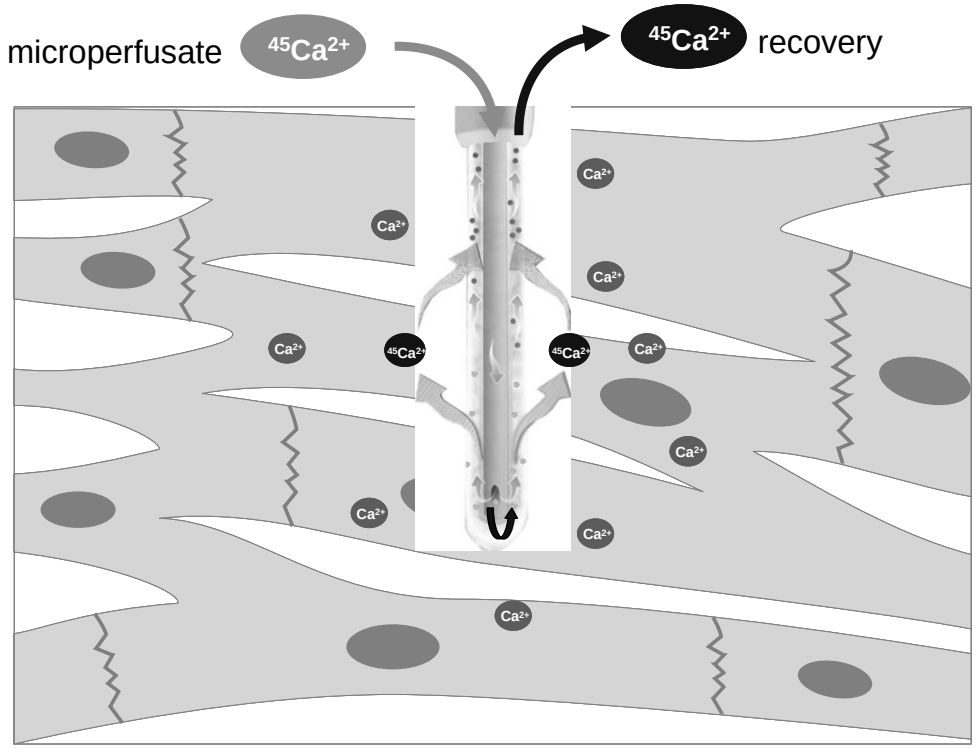

Figure 5. $^{45}Ca^{2+}$ is a marker for calcium diffusion and distribution in the local myocardium. In non-ischemic conditions, $^{45}Ca^{2+}$ follows calcium into cells and then is distributed in the cell's intracellular calcium pool. This distribution of the marker ($^{45}Ca^{2+}$) into cellular compartments contributes to lower 'recovery' during non-ischemic conditions. When cardiomyocytes become ischemic, and intracellular calcium overload ensues, then at some point the gradient for diffusion into cells decreases and less calcium (including the marker $^{45}Ca^{2+}$ moves into cells). This leads to increased 'recovery' of the marker in microdialysate.

The microdialysis sample measurements represent interstitial concentrations, but one must also consider what the interstitial concentrations represent. They represent the result of local delivery and consumption and for some substances cells are the source of the substance (injury markers in the setting of acute local injury) and for other substances cells are consumers of the substance which has been delivered by arterial inflow, like interstitial glucose. In the case of glucose, there is a limited intracellular source, glycogen, which is not reflected directly by interstitial glucose concentrations. The presumption is, though, that glucose is consumed steadily in the cells during ischemia leading to progressively lower interstitial levels and that the lowness of the level during prolonged ischemia reflects the availability of intracellular glucose when glycogen levels have long since been consumed.

Interstitial lactate concentration is both what is delivered and taken away from the area by capillaries as well as what is produced and consumed by cells. Lactate has long been associated with ischemia, where a rapid and steady rise in lactate in microdialysate over the first 20 minutes of ischemia is typical. After that point, lactate levels do not seem to systematically increase, in our experience. Lactate increase has traditionally been a marker for imbalance between local metabolic demand and supply of substrates and has been used for identification of ischemia, often in combination with pyruvate as a ratio. This ratio has several reasons for use. First, if lactate levels are high in arterial blood, then smaller increases in lactate locally might not be appreciated as ischemia. The relation of lactate and pyruvate, where in ischemia lactate increases and pyruvate decreases, can help distinguish ischemia from lactate production from other sources (Larach DB et al. 2011). Pyruvate has been very interesting in microdialysis cardiac sampling, since it seems to rise in concentration slowly during experimental periods, decreasing as expected during ischemic, but rising during non-ischemic periods, though no researchers have found a coherent explanation for this rise as shown in our own work here and with others (Mantovani V et al. 2006). Glutamate is both consumed and produced by heart muscle and is difficult to use as either an injury marker or substrate index in our myocardial sampling, thought it can be expected that during prolonged ischemia , glutamate probably should change, though both increases and decreases have been reported (Valen G et al. 2004, Bäckström T aet al. 2003). The injury marker glycerol in our myocardial ischemia context is thought to be a product of the activation of phospholipase A2 from intracellular calcium overload during ischemia. The substrate for the enzyme is phospholipid in cell membrane. Interstitial and microdialysis glycerol levels rise rapidly during ischemia and decreases just as rapidly during reperfusion after a limited period of ischemia (around 40 minutes is a common model). We have supplemented the assessment of microdialysis injury assessment with the amino acid taurine (Kavianipour M et al. 2004) which is released in large amounts in myocardial cell injury. In our lab experience, taurine levels peak sometime between 60 and 80 minutes during prolonged ischemia (unpublished data, ischemic preconditioning mechanism group, Umeå University), but begin to rise relatively early in the course of ischemia.

Another assessment of energy status which we have employed is the so-called ‘energy charge’ or relation between absolute ATP, ADP and AMP concentrations (Salem J et al. 2002). For this assessment, tissue samples are taken in vivo and then ground up for HPLC measurement of ATP, ADP and AMP concentrations. This is one way to distinguish different energy metabolic states in injured or recovering heart muscle.

In this large animal model, it is important that the general hemodynamic or circulatory conditions are steady throughout the whole protocol. We collect and report blood pressures and heart rate as indicators of model stability. During

myocardial ischemia and particularly during ischemia in a larger portion of the left ventricle, it is expected that there is a general circulatory response which can include a stress response (heart rate increase) and possible blood pressure changes. Our goal was that the animals had an adequate blood pressure throughout the protocol

Protocols

For Studies 1 and 2, carbon monoxide was administered to achieve a specific dose or effect and this was managed by carbon monoxide inhalation throughout the protocol, but dosing for the loading and maintenance phases using caroboxyhemoglobin (COHb) levels as a guide. A 5 % increase in COHb was the goal for a test group and this was chosen as a dose since it is not considered to be directly toxic in general to the animal.

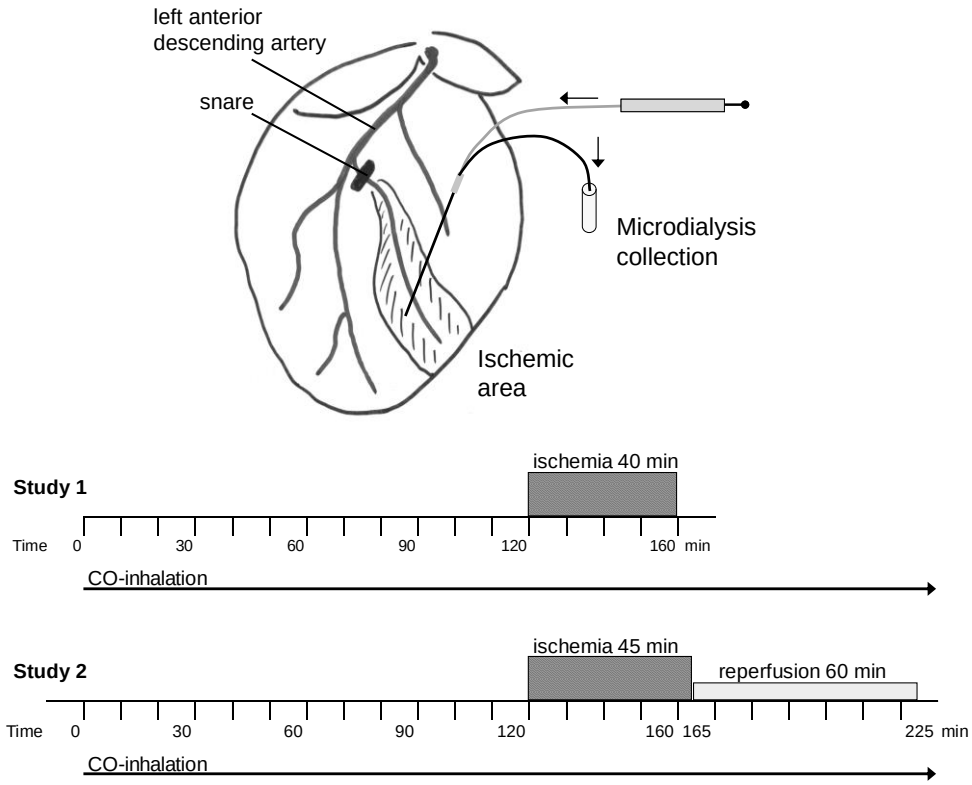

Figure 6. Protocols for interventions and microdialysis collection for Studies I and II.

For Study 1, we conducted pilot studies with a lower dose of carbon monoxide (2.5% increase in COHb, which is half the final level) and we thought that we could have observed response at doses both higher and lower than 5 % increase in carboxyhemoglobin, which supported the choice of the dose that we used. We wanted to avoid doses which might risk making the animals hypoxic. In the literature, related to in vitro studies, some of the doses that were reported were very high in a clinical context (Uemura K et al. 2005, Chin BY et al. 2007). When carbon dioxide binds to mitochondrial cytochromes, there is expected to be an effect on energy metabolism and this is what we aimed to study. Carbon monoxide was given as a pretreatment and it has a very strong affinity to heme molecules. What is studied is to a large extent not technically just pretreatment, since in pretreatment, it is possible for the pretreating substance to be washed out. For carbon monoxide, when the coronary occlusion comes, the carbon dioxide is not washed out, we presume, therefore there is treatment even during the ischemic period.

For Studies 3 and 4, ischemic preconditioning was the pretreatment that was studied. Ischemic preconditioning was delivered in 4 cycles of 10 minutes of snare occlusion followed by 20 minutes of recovery. Then, an index ischemia period was performed. This index ischemic insult is based on the long established infarction model initially made popular more than 20 years ago (Murray C et al. 1986) to study ischemic preconditioning. The length of time for the typical ischemic insult in the index period is that which, in the pig, would bring about an infarction of approximately 50% of the area at risk (Näslund U et al. 1990) and where optimal ischemic preconditioning has been shown to reduce the infarction size by 50%. If the index ischemia time is longer, then the infarction percentage of the area at risk is higher.

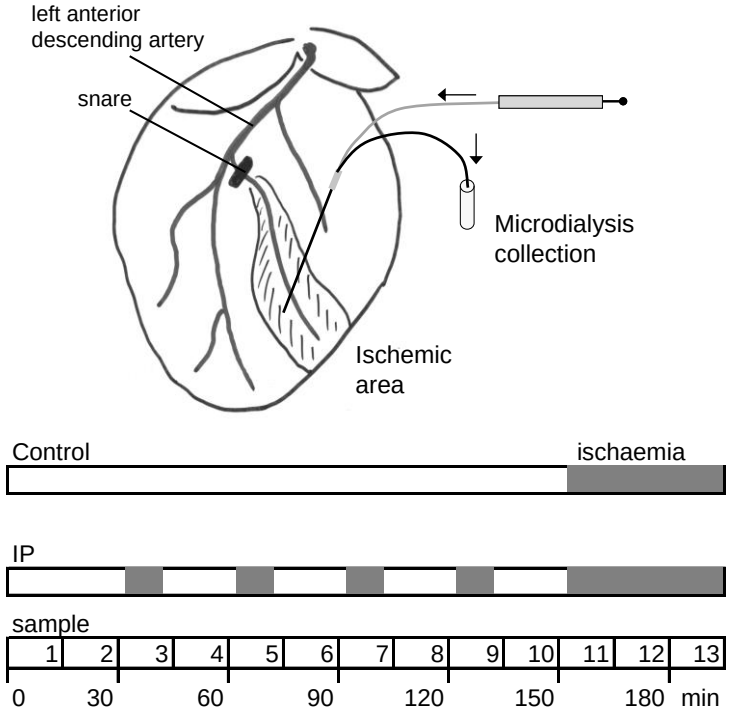

Figure 7. (Study III) . This schematic figure (upper panel) shows the anterior aspect of the heart in situ with a snare and an ischaemic area (shaded). The microdialysis system is represented where Ringer's solution added with ^{14}C-adenosine and glucose in the same concentrations is perfused through the probe and into the local myocardium and samples collected for analysis of ^{14}C-adenosine, the produced ^{14}C-lactate, total lactate, adenosine, glucose, glycerol and taurine. The lower panel shows the experimental protocol; shaded background represents the occlusion of the diagonal branch of the LAD artery, induced by tightening the snare.

If the pretreatment involves delivery of a substance (as with CO) before coronary snare occlusion, then one can assume that there is no more delivery once the snare is drawn and the coronary flow is occluded. Therefore, it must be pre-treatment or post-treatment which is studied if using a coronary occlusion in vivo model, as we have done. In the setting of ischemic preconditioning, it is ischemia itself which initiates a response. With *in vivo* sampling during ischemia, we are able to follow, for selected substances, results of cell uptake or cell production during ischemia. We have focused on this immediate time frame, to try to detect an effect which somehow has led to less energy consumption or a 'resting' state that might confer protection, perhaps even something like metabolic slow-down or hibernation.

RESULTS

Study I

In Study 1, we were able to deliver CO so that there were very precise increases in COHB. In this figure, there is a group with 2.5% COHb increase dose as well as a 5% COHb increase. Carbon dioxide inhalation dose was titrated after co-oximetry results for COHb percent. The lower CO dose was part of pilot work and was not later included in the final analysis and article. Even the lower dose results supported an interpretation that there might be a dose-related response to CO for immediate energy metabolic events during ischemia (see below).

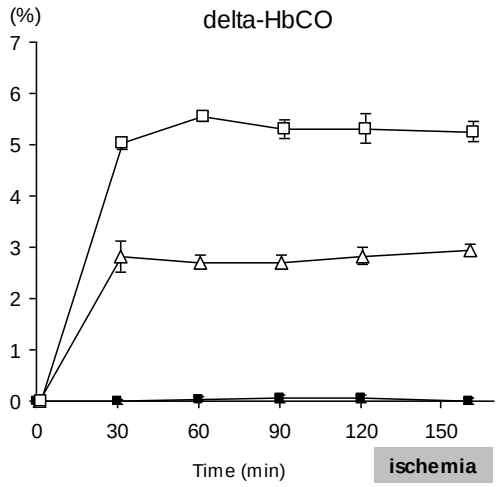

Figure 8. (Study I) The arterial blood COHb changes from start until the end of ischemia in a porcine model with LAD occlusion. Ischemia was induced after 120 minutes of the protocol. Three groups are presented. 5 % COHb over baseline, n=9 (open squares), 2.5 % COHb over baseline, n=6 (open triangles) and controls, n=9 (filled squares). Data are presented as mean ±SEM.

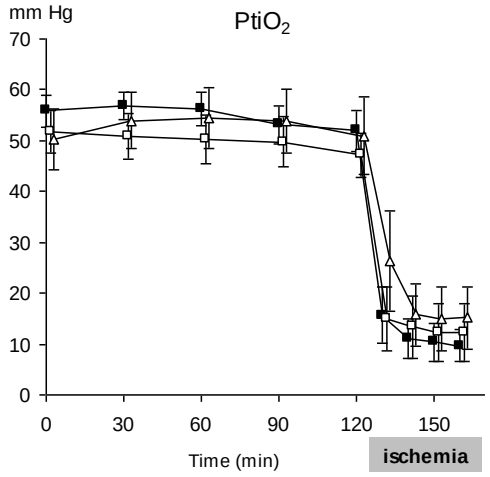

Figure 9. (Study I) - $PtiO_2$ level during the time before and during ischemia, as measured by an electrode for oxygen partial pressure in tissue. Ischemia was induced after 120 minutes of the protocol. Three groups are presented. 5 % COHb over baseline, n=9 (open squares), 2.5 % COHb over baseline, n=6 (open triangles) and controls, n=9 (filled squares).
Data are presented as mean ±SEM.

There was a clear change in tissue partial pressure for oxygen ($PtiO_2$) in the myocardium once the coronary snare was drawn. We initially aimed to use the tissue oxygen tension probe to confirm both ischemia (and reperfusion for the ischemic preconditioning or reperfusion events in later studies). The miniaturized Clark electrode was place in the ventricular wall, as close as possible to the microdialysis membranes, at least so that it could be certain that both were in the area that would be ischemic, though without disturbing each other. There became a problem in using the probe systematically in all the studies, because probes were no longer available for purchase. This is why there are $PtiO_2$ results only for Study I.

There was a clear response in lactate, glucose and lactate/pyruvate during coronary occlusion, where lactate and lactate/pyruvate were higher in the control groups compared to the 5% COHb treated group. Glucose was also lower during ischemia in the control group compared to the 5% COHb treated group. These results combined, suggested a CO effect at least on the production, consumption or elimination of lactate, pyruvate and glucose. No other metabolites were measured in this protocol.

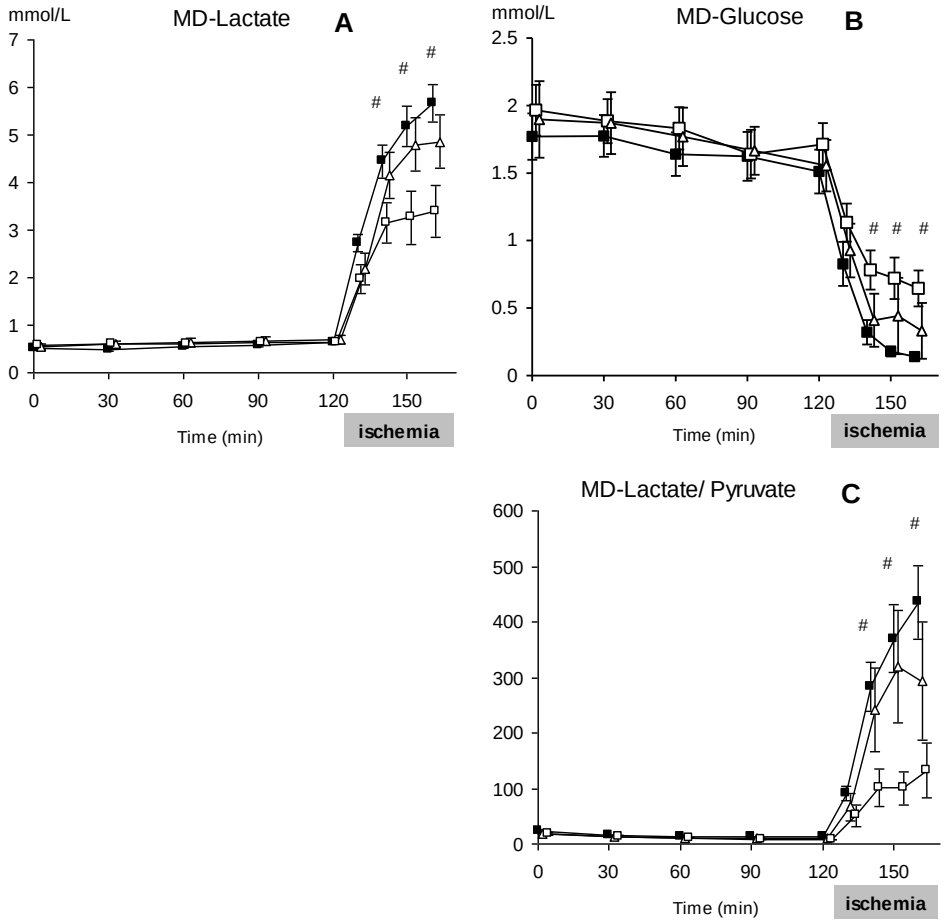

Figure 10. (Study I) Lactate results are shown (panel A) in MD-samples from start and during myocardial ischemia. Ischemia was induced after 120 minutes of the protocol. Three groups are presented. 5 % COHb over baseline, n=9 (open squares), 2.5 % COHb over baseline, n=6 (open triangles) and controls, n=9 (filled squares). In panel B, glucose results for the same microdialysis (MD) samples are show, as are those for Lactate/Pyruvate ratio (panel C). CO pre-treatment had effects (#) on lactate, glucose and pyruvate (lower panel) during the ischemic myocardial injury. CO appeared to influence pyruvate levels, compared to controls, even during the pre-treatment period. (see Study I Results). Data are presented as mean ±SEM. # $p<0.05$ control group vs. 5% COHb group using T-test between groups.

The 2.5% COHb treated pilot results are shown as well in the figure, though these were interpreted as not differing from 5%, which is why we decided to not have multiple but near CO doses for the final study (we did not redo 2.5% COHb experiments once the protocol was set, and we did not include the pilot 2.5% COHb results in the final paper).

Purine nucleosides were measured for the same experiments, were we could not demonstrate difference in energy charge related to CO. Differences were shown for energy charge, as expected, between ischemic and non-ischemic tissue.

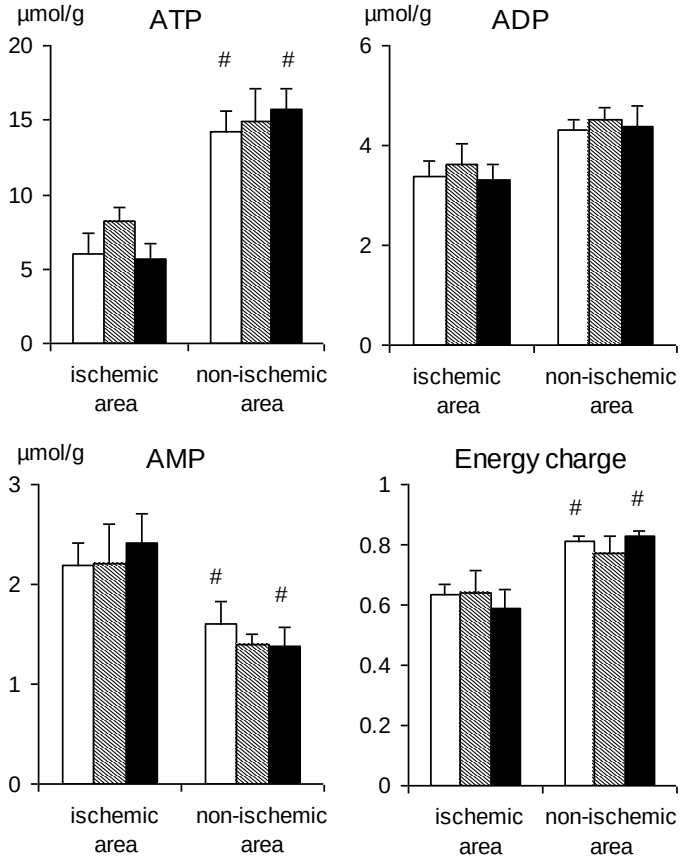

Figure 11. (Study I). Results for adenosine tri-phosphate (ATP), adenosine di-phosphate (ADP) and adenosine mono-phosphate (AMP) along with and energy charge, calculated as Energy charge = (ATP + 1/2ADP) / (ATP+ADP+AMP) are shown for biopsies in ischemic and non-ischemic left ventricular free wall taken during the same minute at the end of the protocol.
Data are presented as mean ±SEM, Three groups are presented. 5 % COHb over baseline, n=9 (open bars), 2.5 % COHb over baseline, n=6 (hashed bars) and controls, n=9 (filled bars).
# $p<0.05$ using paired T-test, non-ischemic versus ischemic areas after 40 minutes of ischemia.

Study II

Based on the results from the first study, we felt that there was reason to test CO for effects on early injury markers which could be collected during the ischemic period in a similar protocol. We expended the period for testing to include a reperfusion time with multiple samples. We also included several injury markers (glycerol, $^{45}Ca^{2+}$, and glutamate) which are not direct indicators of coming infarction, but could add information concerning immediate energy metabolic conditions. The lactate accumulation results which supported the idea of a CO effect during ischemia were recognized as potentially multifactorial in origin, and not necessarily due to a CO-related energy metabolic mechanism. Therefore, we proceeded with a more in-depth and more specific Study II, including additional measured metabolic and injury parameters.

One finding shown in the Study II publication was that there was no difference in lactate and pyruvate indicators between CO treated and non-CO treated groups during ischemia and immediate reperfusion periods for this series. Combining data from Studies I and II illustrates (Figure 12) that there is quite a bit of variability in lactate levels (lactate accumulation in tissue) during ischemia, which suggest that there are likely several independent processes which can have influence on the resulting lactate accumulation in tissue during ischemia.

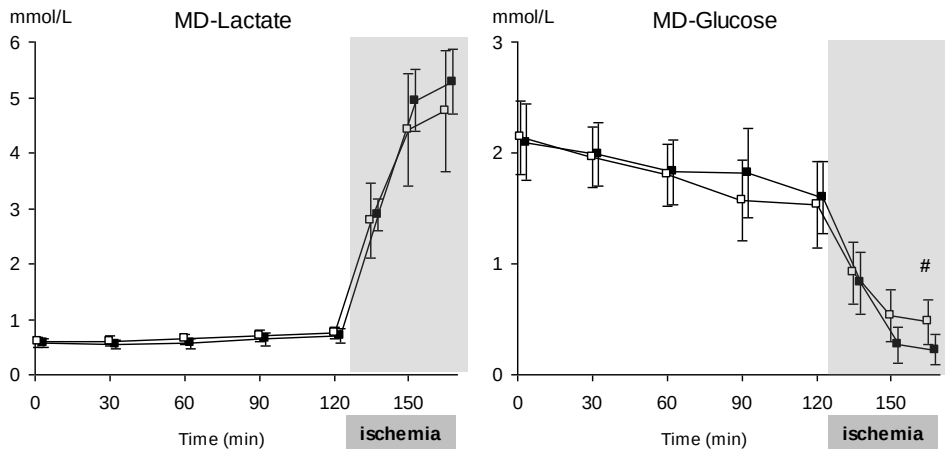

Figure 12. Pooled results from Study I and II. Mean ±95% confidence intervals, n=17.

Results for $^{45}Ca^{2+}$ recovery and glycerol (Figure 12) did not indicate a metabolic difference during ischemia associated with CO treatment (5% COHb increase). There were no indications of differences during the immediate post-ischemic period. These results awakened questions about whether the dose of CO was optimal for demonstrating an energy metabolic or injury effect, or if injury detection needed to be conducted in another time frame (perhaps much later).

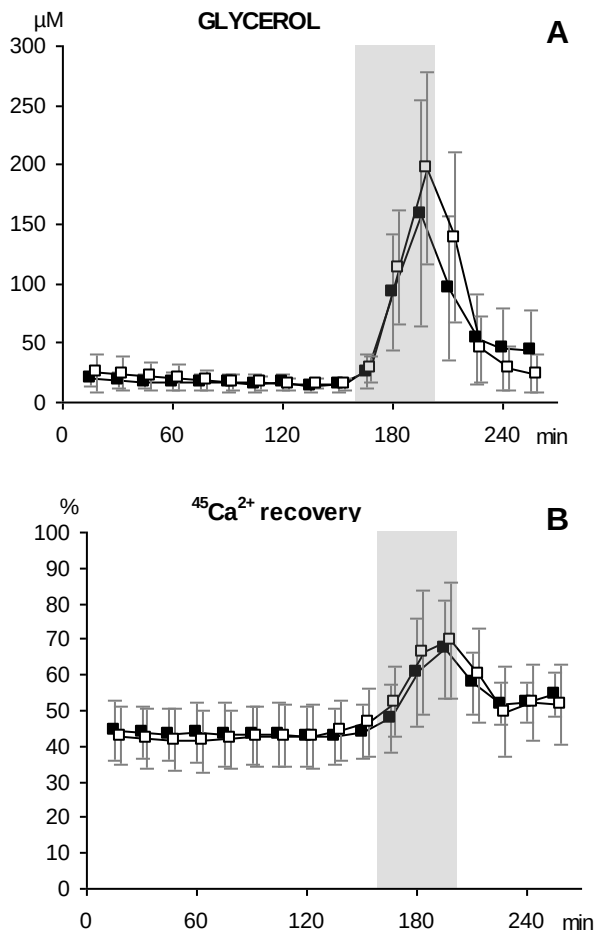

Figure 13. (Study II) This figure shows the protocol for both groups, with CO administration starting at 30 minutes (treated-open squares, n = 8, with control observations in filled squares, n = 8, (95% CI), then coronary occlusion (shaded portion) and reperfusion. Ischemia was associated with increases in local glycerol and less local myocardial calcium uptake as shown by radio-marked calcium recovery increases and these reverted towards normal during reperfusion. There were no differences between CO-treated and control groups for these parameters.

Study III

We continued our study of energy metabolism during ischemia with another proven protective pretreatment, that is, ischemic preconditioning. The study design here used the idea that radio-marked adenosine, if converted by the enzyme purine nucleoside phosphorylase (PNP) ultimately to ribose-1-phosphate, could enter the glycolytic process as an energy source and that this could be assessed by detecting an quantifying the same radiomarker in lactate molecules (see diagram below).

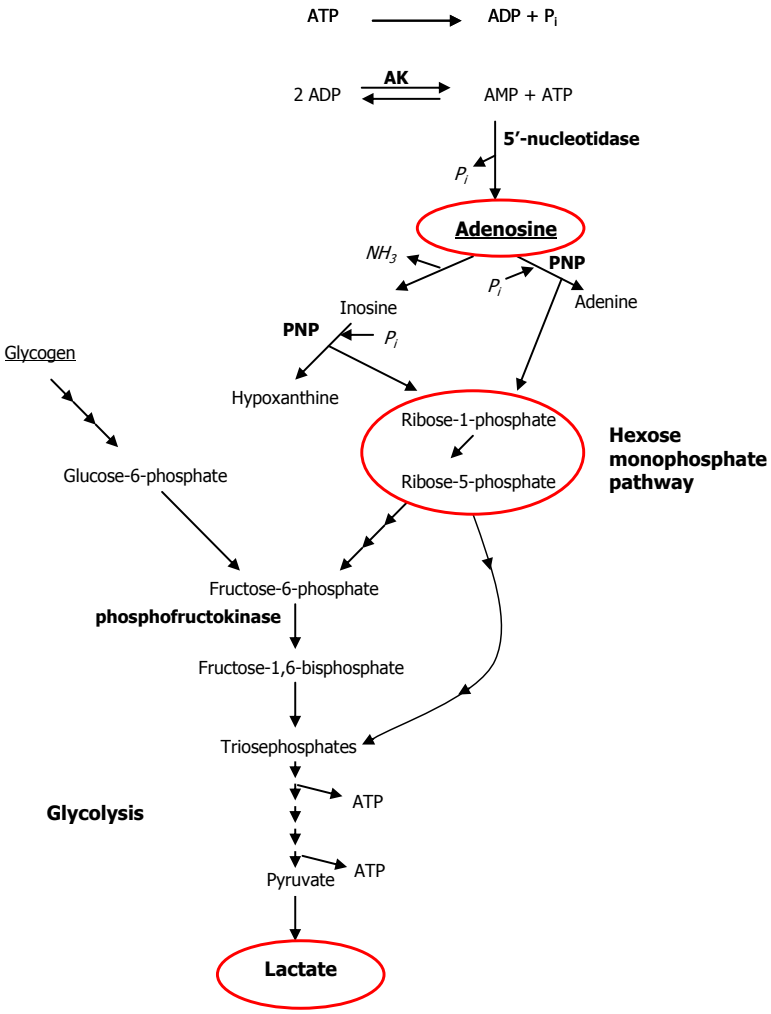

Figure 14. (Study III) Figure A. Adenosine, the hexose monophosphate pathway and glycolysis. Schematic illustration of the catabolism of adenosine and glycogen. ATP is degraded to ADP and AMP during ischaemic conditions, resulting in an accumulation of AMP. AMP is then removed by further metabolism to adenosine and in a second step, inosine. PNP metabolises inosine to ribose-1-phosphate which then enters the glycolytic pathway and regenerates ATP. This entry of substrate into glycolysis occurs even when glucose can no longer be liberated from glycogen due to ATP shortage.

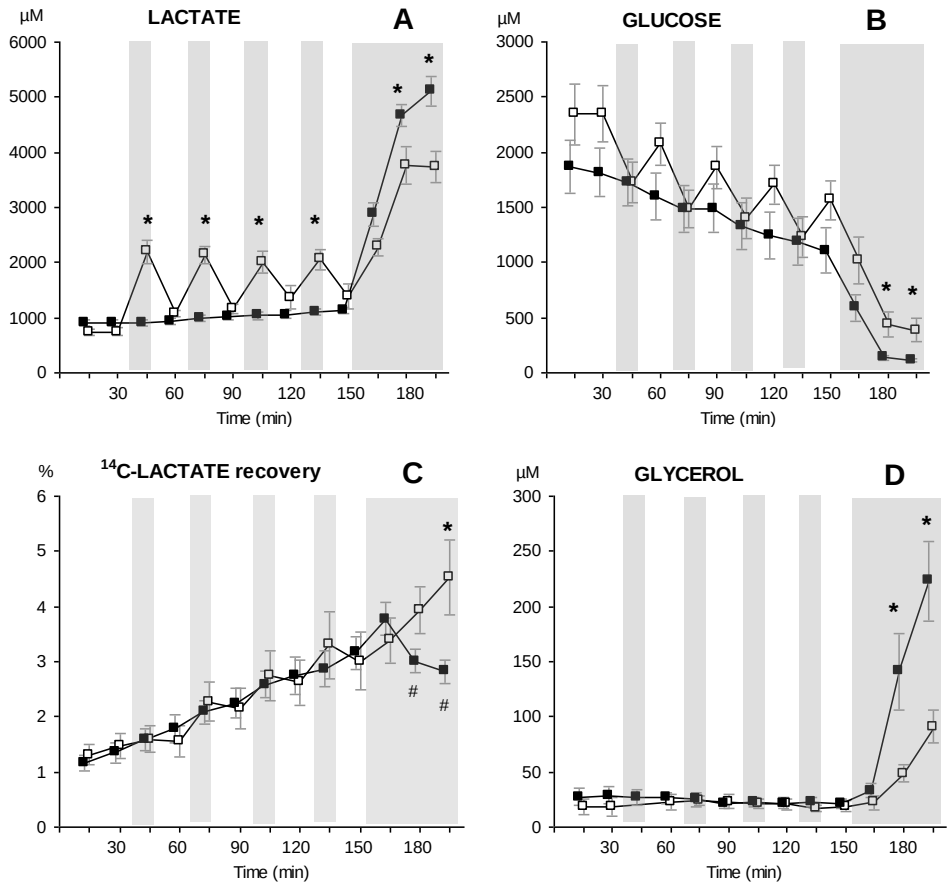

Figure 15. (Study III) Lactate (panel A), glucose (panel B), and glycerol (panel D) concentrations together with ^{14}C-lactate recovery (panel C) in MD samples (mean ± SEM) from the IP (open symbols) and the control (filled symbols) group. N=7 for IP and n=8 for controls except for glycerol (IP n= 5, control n=6). Coronary occlusion is represented by shaded background and the control animals were not subjected to any ischaemic episodes prior to the prolonged ischemia. Differences between groups are shown by * and difference within group during the prolonged ischemia are shown by #, $p<0.05$.

We found that ischemic preconditioning cycles before a prolonged ischemia could be observed if measuring interstitial lactate (Fig. 15A) or glucose (Fig.15B). There was less lactate produced in general but more lactate produced from adenosine (^{14}C-lactate, derived from ^{14}C-adenosine) in the ischemic preconditioning-treated animals. This was associated with less release of the injury marker glycerol, as expected. We interpreted the higher glucose levels and lower lactate levels in general, to mean that adenosine being used as a metabolic substrate influence amount of glycolysis that occurred and that this is occurring at the same time that there is a protective influence in the same cells. The PNP enzyme seems to have

activity even without ischemic preconditioning, but the major effect that we were looking for occurs during the prolonged ischemic period, where PNP activity seems to drop off in the untreated but ischemic cells compared to acceleration in the ischemic preconditioned cells (Fig. 15C). How this occurs and if it is solely related to PNP activity became a question for the next study.

We also measured tissue glycogen levels for ischemic preconditioned or untreated animals, before and during ischemia. We found the expected decrease in glycogen levels for both groups during ischemia, but no clear difference in glycogen levels related to ischemic preconditioning (figure 15).

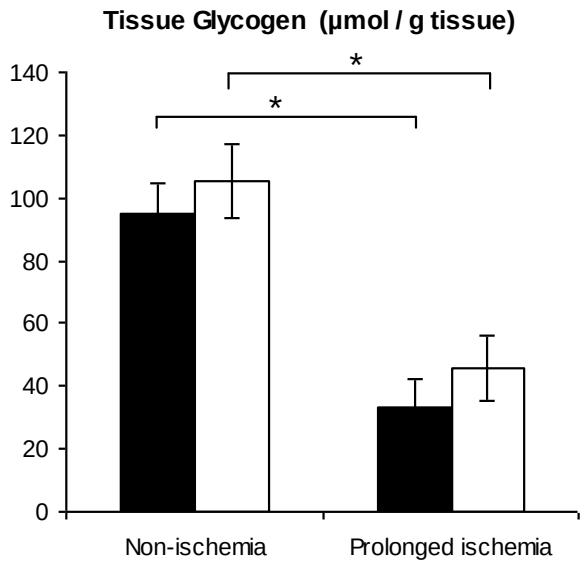

Figure 16. (Study III) Tissue glycogen (after enzymatic hydrolysis to glucose) from biopsies (IP, white bars, n=7; control, black bars, n=8; mean ± SEM) show approximately 60% less glycogen content at the end of the prolonged ischemia compared to non-ischemic tissue. There was no difference between the IP and control group for glycogen content at the end of the prolonged ischemia.

Study IV

In this study, we delivered PNP blocker locally to ischemic preconditioning treated animals, to see if very strong inhibition of PNP would lead to an inhibition of the protective mechanism which limited injury marker release in preconditioned tissue. We used 2 separate catheters to deliver PNP blocker or not (and then sample) and these were separated by at least a centimeter so that there would be no

cross-influence between probes as far as diffusion of blocker. The energy metabolic markers (figure below) show clear ischemic reconditioning responses for both groups as far as lactate, pyruvate and glucose.

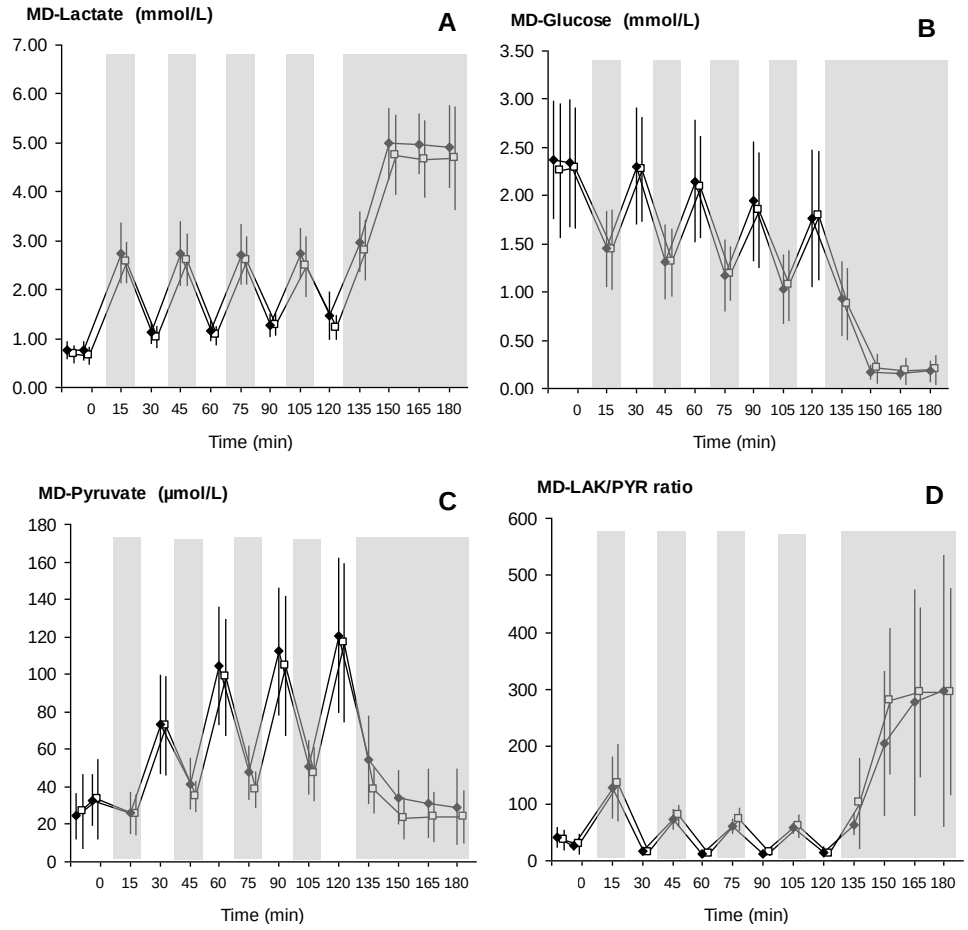

Figure 17. (Study IV). Data are presented as mean ± 95% confidence intervals (n = 8). Filled diamond = PNP-blocker; open square = No PNP-blocker. Ischemic preconditioning cycles are shown by the 4 narrow shaded areas, and the index (60 minute) ischemia is shown by the wide shade area during the last part of the protocol. There were no significant differences between groups for these parameters, with grouped t tests performed for Control (0 minutes), pre-index ischemia (120 minutes), and the last 2 points during index ischemia (165 and 180 minutes).

At the same time, adenosine metabolism as an energy source seems to be practically eliminated by PNP blocker delivered this way (figure below).

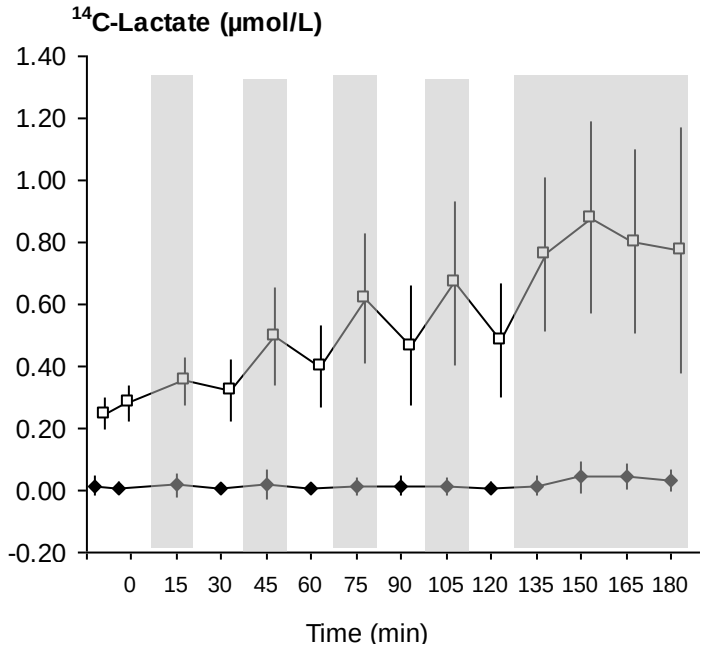

Figure 18. (Study IV) . Data are presented as mean ± 95% confidence intervals (n = 8).
Filled diamond = PNP-blocker; open square = No PNP-blocker. PNP blockade delivered locally by microperfusate effectively blocks local cardiomyocyte adenosine utilisation for energy purposes, even during prolonged ischemia.

This did not lead to a difference in injury markers related to blocking of PNP activity during ischemic preconditioning and prolonged ischemia (Figure 19). We would have expected relatively lower levels of injury markers in the ischemic preconditioned animals where PNP was activated, if PNP was central in the protection process.

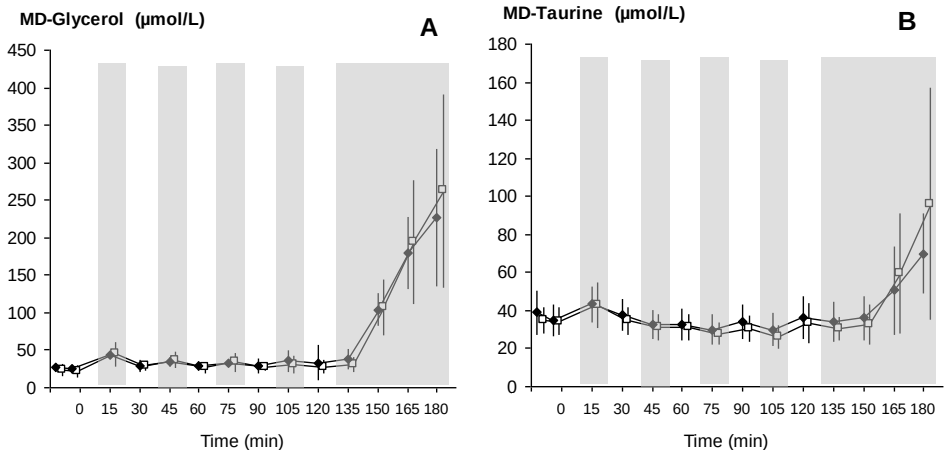

Figure 19. (Study IV). Data are presented as mean ± 95% confidence intervals (n = 8).
Filled diamond = PNP-blocker; open square = No PNP-blocker. Glycerol and taurine increase in both groups during the index ischemia period, though there is no difference between groups related to PNP blockade.

DISCUSSION

These projects have been focused on the energy-metabolic conditions associated with myocardial ischemia, together with effects of pretreatments which have been demonstrated to be protective for myocardial tissue, though through as yet unclear mechanisms.

We have designed these studies to examine the immediate conditions during ischemia, in order to try to detect if energy metabolic conditions are influenced by these interventions. We have limited the scope of these studies to the immediate ischemic period. When we consider protective effects from an ischemic insult, these are usually measured after many hours by quantifying infarction. The actual steps or events which limit the later infarction amount or degree may be a signal cascade or an event which occurs later during reperfusion and recovery. Injury can lead to infarction or to increased apoptosis. These are later events. The exact mechanisms are not known. The focus here was to try to see if immediate energy conditions during ischemia were involved. This could not be shown clearly in the carbon monoxide experiments, though there was a hint in Study I that carbon monoxide may have some effect on immediate energy conditions during ischemia. There is clearly an energy metabolic effect of ischemic preconditioning as viewed when measuring glycolysis and we took a step towards trying to identify a specific enzyme system activity (PNP) as necessary or not for this effect, though these first results are not at all conclusive.

There is no doubt that carbon monoxide has an effect on cellular respiration, even if low levels do not impair oxygen delivery to cells. It is possible that carbon monoxide has many other effects which are relevant to cardiomyocyte function which are not appreciated now. Carbon monoxide may have effects which are first seen many hours later and on cells of other types than cardiomyocytes. Apoptosis may be relevant as far as carbon monoxide effect. Inflammation modulation is clearly also a possible effector system for CO. These types of late effects may be very relevant for clinical application, but are not part of the immediate energy metabolic events that we have measured.

Our analysis of the metabolic role of CO in myocardial energy-metabolism and protection was performed in 2 steps. First, in Study I, we explored the most readily accessible but less specific parameters which microdialysis can offer, which were lactate and pyruvate. Even before Study I, we had done some dose-testing (shown in the results section of this thesis), though we had settled on a potentially clinically relevant dose to test. We acknowledged during Study I that lactate accumulation is multifactorial in origin and that lactate by itself might not be specific enough to draw definite conclusions about degree of ischemia or injury, but the findings still motivated us to pursue this issue in a more in-depth study. In Study II, we examined CO effects in the ischemia model using not only injury markers in addition to the glycolytic parameters, but also including a novel method

for assessing cellular energy status during ischemia ($^{45}Ca^{2+}$ recovery). This allowed more specific evaluation of CO-associated metabolic events.

Myocardial protection from ischemic preconditioning has also has traditionally been assessed using measurement of infarction percentage of area at risk and also tissue samples from the later recovery period to describe cellular events. A clear connection between immediate energy metabolic conditions and tendency for survivability after a limited ischemic period has not been shown yet and this was our focus. There is no doubt that glycolytic conditions during a later index ischemia are affected by recent cycles or burst of short ischemia. This must not only have influence on cell energy resources, but also activate signals within the cell related to oxidative and other forms of stress. Local inflammation could be stimulated and this can have direct or indirect effects on cells. These types of events can clearly increase of the hours following a limited but lethal ischemic period (lethal for some of the cardiomyocytes) and the process following the index ischemic period can be very important. Much is known about the signaling cascades during reperfusion which lead to a lethal event, such as mitochondrial transition pore opening (Murphy E at al. 2010, Murphy E at al 2008).

It is still not known why one cardiomyocyte die from an ischemic insult and a neighboring cardiomyocyte survives. Our results have shown some promise that there may be energy metabolic differences between these cells, but still we have not found conclusive findings to support an argument that this is absolutely the case.

METHODOGICAL CONSIDERATIONS

A strength of the study design that we have employed here is that we have tested energy metabolic events in an in vivo situation in an animal model which closely resembles the human heart (Heusch G et al. 2011), though these healthy young animals with acute experimental preparations have healthy hearts. This is a strong step for translation of results which have demonstrated promising protective interventions in cell or even in rodent models, but cannot be directly tested yet in the clinical setting. The model is highly invasive and this is necessary in order to have a very carefully controlled ischemic event together with reliable localized sampling of the portion of the heart which is ischemic. We have combined localized sampling during ischemia with localized tissue sampling for later analyses for quantification of energy metabolic compounds.

We have also used a very localized means of delivering radiomarked substrates or indicators (ions) to exactly the area which is being studied. With this study design aspect, we have been able to use microdialysis to both deliver substrate or marker and then also sample the results of this delivery over time. This has been a methodological advance which is very promising for future studies. In the case of ^{14}C-marked adenosine, the marker is very expensive, and it would be unrealistic to design a study which required large amount (whole organ). For this, delivery of the substrate locally to cardiomyocytes in microdialysate, which also were sampled over a study period (same catheter) is very effective. Concerning $^{45}Ca^{2+}$ ('recovery') as a marker for calcium overload in ischemic heart tissue, the concept has been demonstrated previously (Engström I et al. 1995), but this is also a methodological advance to be able to measure this in exactly the chosen place in situ in controlled coronary occlusion.

Our study design has limitations. In retrospect, it would have been optimal to prolong all the experiment and perform infarction quantification experiments with infarction percentage of area at risk. We chose to begin with these immediate ischemia period assessments, and possibly, depending on the results, proceed later to infarction quantification. It is not clear that if we had infarction quantification results, which reflect also very much the reperfusion phase, that it would have been easier to interpret these results.

It is possible that what we have measured here, in general aspects of glycolysis and injury markers, are not reflecting the events we are trying to assess. Glycolysis may proceed to some extent, in cells during ischemia, based on factors which are not related to cell survival or later necrosis. There may be other factors which guide both glycolysis and cell survival which we have not taken into account in this study design. Thus, it may be other factors and markers that we need to examine in order to test our hypotheses better.

Microdialysis has the advantage that it is very localized, and can be directed to exactly the organ and tissue area of interest. It also has the disadvantage of repre-

senting very localized sampling. If there is significant heterogeneity in the metabolic activity of the organ region of interest, then microdialysis could be sampling a very small region which does not reflect the same regional result that we aim to study. It is known that in an ischemic area, if the ischemic insult is of a moderate degree, then some myocardial cells will not survive and others will survive. This indicates some degree of heterogeneity (even if small in degree) in an ischemic myocardial area. It is not clear if there are clear differences locally as far as coronary blood flow once a coronary occlusion is complete, as in our model. There is definitely a boundary or 'penumbra' zone where there is some collateral coronary flow and where the cells may have some decreased supply of substrates but not the same as complete ischemia. We have done all that we can to ensure that we are sampling complete ischemia, though there still may be some small differences in some way from point to point in the ischemia zone. This is not well studied and has been the focus of another project in our research group.

Microdialysis is also quite complex as a tool for sampling of cardiomyocyte activity, since it basically collects substances from a particular location in the interstitium, and does not sample cell activity directly. These substances can move in and out of cells and move in and out of capillaries. The end result in the microdialysate reflects only the concentration of substance in the interstitium at the time of the sampling. Some of the substances that we have followed are both produced and consumed by cells. Therefore, there is a degree of interpretation based on context required. When there is a coronary occlusion, then it is assumed that there is a severe limitation in the amount of new delivery (as well as removal) of substances from the local capillaries. When there is ischemia and local hypoxia as well as limitation of glucose supplies, then we assume that cells continue to take up glucose and that there is a conversion to anaerobic metabolism. There is some cellular lactate consumption in aerobic conditions generally, but what is seen as lactate concentration in the interstitial fluid (and microdialysate) represents the result of anaerobic metabolism and lactate production from glycolysis. The same goes for glutamate.

There are a number of possible sources of variability in microdialysis results. One possible source of error can be sub-optimal probe and membrane placement. The probe is not sampling pure ischemia when the coronary snare is drawn. Despite our efforts to place the probe in the middle of the ventricular wall in the ischemic area, sometimes the probe membrane does not end up in the ideal place. Another possible problem is poor exchange over the microdialysis catheter membrane. The membrane can be injured, or for some reason not have optimal contact with interstitial fluid and interstitial substance fluxes. We have been careful to evaluate the responses in microdialysate to ischemia (rapid glucose drop and lactate rise in response to coronary occlusion). If the response is not consistent with immediate response to coronary occlusion and ischemia, then we have not analyzed the results from that catheter further. There was some dropout of

catheters and results, which is why we always tried to use multiple catheters and sampling of the same area for these costly experiments. When we have systematically had multiple catheters sampling the same area and providing results, then we have combined the catheter results to give one data point. Since microdialysis is such a localized sampling method, the ideal way to utilize it would be always to have multiple catheters and multiple sampling to describe an area or region.

The clinical implications of these results are open to discussion. Carbon monoxide (clinical inhalation or by other means) has not reached clinical trials yet, to my knowledge. These studies were of a nature more investigating the mechanism of effects from carbon monoxide on ischemic heart cells. The findings probably do not bring us much closer to a clinical implementation of carbon monoxide as therapy. Neither have these findings demonstrated any clear negative effects, at least for the chosen doses that we studied, of carbon monoxide during ischemia. One possible scenario is that higher doses of carbon monoxide could provide more obvious protection during acute ischemia, but that there are clinical effects of the carbon monoxide itself for the organ which are by themselves possibly injurious. These studies tested only one dose of carbon monoxide. It was a dose that could conceivably be clinically relevant, at least as far as not by itself being toxic.

The ischemic preconditioning studies were also examinations of mechanisms of action and by themselves probably do not have significant clinical implications. Ischemic preconditioning is already an established principle in clinical practice, and in many settings is implemented routinely (for example as a brief pre occlusion treatment before temporarily stopping the circulation to a vital organ (for example, heart or kidney). Our goal is to elucidate mechanism of action so that the same type of protection could be used in other settings. If there was a mechanism involving activation of a specific enzyme system or based on a specific second messenger, then this activator (if we know what it is and can isolate it) could be brought to another situation where one knows ahead of time when there is going to be an injurious ischemic period (transplantation, donated organs, for example) and potentially provide a similar degree of protection as ischemic preconditioning. As things are, there can be some hesitation to make an organ clearly ischemic as part of a treatment plan, even if the goal is protection, since there can be significant complications even with limited ischemia. Our ultimate goal is to find energy metabolic related mechanism with these types of (potentially threatening) pre-treatments, so that possibly a new type of drug therapy can be generated which could initiate the protective results without the initial insult. This is a long-term goal, and these studies were steps in that process.

FUTURE DIRECTIONS

Future directions for this research may include testing other doses of carbon monoxide and other time frames for measuring injury. For ischemic preconditioning, we can continue to study the role of the activation of the PNP enzyme system as well as different means to activate the enzyme (other than ischemic preconditioning). We will need to establish if blocking the PNP enzyme eliminates all the protective effects of ischemic preconditioning in an infarction quantification model. I definitely think that further study of these questions in a pig model are correct, since the pig heart is so close to the human heart in structure and physiology (including response to ischemia). Through this work, I hope to grow as a researcher.

SUMMARY AND CONCLUSIONS

Summary of the findings

1. The hypothesis-generating findings in Study 1 were that there was an indication of effect (reduction/limitation) on interstitial concentrations of glucose and lactate, lactate/pyruvate related to CO pre-treatment (5% increase in carboxyhemoglobin percent) in this porcine in vivo myocardial ischemia model.
2. In the subsequent expanded metabolic and injury marker examination of CO in this same dose (Study II) before prolonged ischemia, CO was not associated with an ameliorating effect during the 45 minute of ischemia as far as the immediately released glycolytic and injury markers in the porcine *in vivo* myocardial ischemia model.
3. Ischemic preconditioning led to enhanced radio-marked adenosine consumption as an energy-metabolic substrate, together with slower glycolytic flow (as less glucose consumption and lactate formation) in ischemic preconditioned heart muscle.
4. Local purine nucleoside phosphorylase blockade inhibited adenosine utilization as an energy-metabolic substrate during ischemia, but this did not have a clear effect on glycolysis or injury markers during prolonged ischemia after ischemic preconditioning.

Conclusions

1. Pre-treatment by CO in this dose (5% increase in carboxyhemoglobin percent), administered to the heart in an *in vivo* porcine model, has no demonstrable effect on glycolysis or immediate injury marker release during prolonged (40 or 45 minute) ischemia.
2. Carbon monoxide protective effects in other doses or time frames cannot be ruled out.
3. There may be an immediate energy-metabolic explanation and mechanism, with adenosine consumed during ischemia as an energy substrate during prolonged ischemia, for why more ischemic preconditioned cells survive after prolonged ischemia, but more study is needed.
4. Experimental purine nucleoside phosphorylase blockade appears to allow interruption of ischemic preconditioning-related adenosine utilization as an energy-metabolic substrate during prolonged ischemia without obvious effects on glycolysis, and this requires further study to test if this adenosine energy resource during ischemia is associated with protection during infarction.

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Populärvetenskaplig sammanfattning på svenska

Hjärtat är ett fantastiskt organ. Varje sekund, varje dag, genom hela vårt liv arbetar det konstant. Men detta konstanta arbete gör det också sårbart. Det är beroende av att hela tiden få olika ämnen till sig och avfallsprodukter borttransporterade för att kunna fortsätta att arbete. Transporten sker via blodet och ett oavbrutet flöde av detta ger hjärtats celler tillgång till syre och olika näringsämnen för att kunna skapa energi samt transportera bort avfallsprodukter såsom koldioxid. Om blodflödet avbryts kommer hjärtats celler snabbt att få brist på energi och från att ha föredragit att använda fettsyror som energikälla så kommer energin nu framförallt från glukos. Detta tillstånd kallas anaerob metabolism (utan syre) till skillnad från aerob (med syre). Det är mycket mindre effektivt och ger mycket mindre energi, men räddar cellen endast under en begränsad tid, innan blodflödet kommer igen. Cellen kommer obevekligen att dö om inte detta sker, men hur länge cellen kan överleva följer en flytande skala. Efter en kort tid utan blodflöde repar sig de flesta celler. Förlängs denna tid så överlever färre och färre celler i hjärtat för att till slut dö allihopa. Detta är vad som sker vid en hjärtinfarkt, ett tillstånd som ger ett mycket stort antal dödsfall i västvärlden.

Skulle det kunna vara möjligt att förlänga den tiden som man har på sig från det att blodflödet upphört tills det är utom möjlighet för cellen att repa sig? Då skulle det finnas större chans att hinna återställa blodflödet, t ex med en bypass operation.

I mitt arbete har vi studerat vad som händer med metabolismen (ämnesomsättningen), när blodflödet stryps, ett tillstånd som kallas ischemi, och följt förändringar med mikrodialys. I två arbeten har vi använt kolmonoxid för att klargöra om detta ämne har en gynnsam effekt på energimetabolismen. Kolmonoxid är ett ämne som har fått mycket uppmärksamhet, från att tidigare betraktats som ett enbart giftigt ämne, nu bedöms verka som signalmolekyl. I de två följande arbetena använde vi ischemisk prekonditionering, som kan beskrivas som korta ischemiperioder med efterföljande reperfusion, som var och en inte ger upphov till permanent skada på celler, men tycks verka gynnsamt om dessa perioder efterföljs av en längre period med upphävt flöde. Vi studerade adenosinets roll för energimetabolismen samt ett enzym kallat purin-nucleosid-fosforylas.

Samma modell för hjärtinfarkt, på sövd gris, har använts i alla arbeten. Grishjärta används för att det är mycket likt det mänskliga unga hjärtat. Varje gren av blodkärl försörjer sitt område av hjärtat med blod och det finns knappt blodflöde från angränsande kärl, alltså kan man lätt skapa ett område med infarkt. Inför försöket sövs grisen med noggrann övervakning. Bröstkorgen öppnas för att komma åt hjärtats kärl och runt en kärlgren läggs en snara, som när denna dras åt stoppar blodflödet i kärlet. Mikrodialyskatetrar läggs in i hjärtmuskeln. Man kan även tillföra ämnen via mikrodialyskatetrarna.

I mitt första arbete används infarktmodellen för att studera om kolmonoxid ger någon påverkan på hjärtcellens metabolism som är möjlig att följa via mikrodialys, när det tillförs innan och under ischemi. Kolmonoxidet tillfördes via respiratorn och hur mycket som skulle tillföras titrerades via blodprov och vi jämför med en grupp som inte fick någon extra kolmonoxid tillförd. I bägge skapade vi ett hjärtinfarktområde under 40 minuter. Vi såg att under ischemiperioden fick vi kraftig ökning av laktat i båda grupperna, vilket betyder att vi hade strypt blodflödet, då laktat bildas i ökad mängd vid anaerob metabolism. Men vi såg också att i gruppen som fått kolmonoxid så blev ökningen av laktat lägre vid

strypt blodflöde. Kunde detta tyda på att det finns en cellskyddande effekt?

I andra studien har jag med samma modell studerat ämnen som bildas vid metabolism, och nu lagt till analys av glycerol, ett ämne som kommer från cellens membran och som vars utsöndring ökar då cellen börjar gå sönder och glutamate, en aminosyra som finns intracellulärt. Ytterligare en metod för att analysera cellens energitillgång användes också. I cellens membran finns jonkanaler som pumpar calcium. Detta är energiberoende så vid energibrist blir det ökad mängd calcium intracellulärt och om calcium tillförs utifrån så kommer detta inte att kunna tas upp intracellulärt. Det här har vi använt på så sätt att vi tillförde en isotop av calcium i mikrodialysvätskan och vid energibrist så kommer mindre av detta calciumisotop att tas upp. Vi får därför högre nivå på det radiomärkta calciumet i våra prover vid energibrist. Förutom dessa utökade analyser så lade vi även till reperfusion, dvs öppnade upp blodflödet igen, under 60 minuter. Det vi fann var att laktat ökar under tiden snaran stryptes och sjönk igen när blodflödet återkom. Det tyder på att vi uppnådde ischemi i önskat område men vi såg ingen skillnad i laktat mellan grupperna denna gången. Övriga ämnen tydde på att det var en anaerob metabolism när snaran ströps med tecken på cellskada samt minskad tillgång på energi, men ingen skillnad mellan våra två grupper. Vi tolkar laktat som bra för att bedöma att man har anaerob metabolism, men att det inte är användbart för att tolka total metabolism och energitillgång. I vårat försök, som innebar en relativt låg dos kolmonoxid, men en dos som valdes för att den i tidigare arbeten tycktes ha effekt, samt var tillräckligt låg för att inte ge toxiska symptom, samt en kort exponeringstid, så verkar inte kolmonoxid ge någon gynnsam effekt som vi kan mäta.

I det tredje arbetet ville vi närmare studera ischemisk prekonditionering och dess påverkan på energitillgången. Det är ett välkänt fenomen och det innebär att man gör korta episoder av avstängt blodflöde med återställt flöde däremellan och varje avstängning är så kort att man inte får en permanent cellskada. Adenosine bildas när cellen använder ATP, som är dess energirika molekyl. Adenosin metaboliseras vidare till laktat och om möjligheten för adenosin att metaboliseras ökar med prekonditionering, kunde det förklara det avtagande mönstret. Vi ville använda vår grismodell för att vidare studera detta. Då det är möjligt att även tillföra ämnen via mikrodialys så gjorde vi så att vi märkte adenosin med en radioaktiv isotop och detta gav vi i våra katetrar tillsammans med glucos. Vi såg att i den gruppen som hade fått prekonditionering så ökade laktat som var isotopmärkt och glukos sjönk inte så mycket. Vi tolkade detta som att cellerna fick lättare att använda adenosin för energi. Då glucos inte sjönk så mycket så bedömde vi detta bero på att glucos inte var den energikälla som föredrogs då det fanns adenosin och prekonditionering skett. Varför blir det så? Enzymet som metaboliserar adenosin vidare till laktat är enzymet purin nucleoside fosfatas (PNP). Det är verksamt på låg nivå hela tiden men möjligen uppreglerades dess aktivitet av vår behandling. Vi ville studera enzymets betydelse och detta föranledde arbete fyra.

Vi använde ånyo vår grismodell. I det blivande ischemiområdet placerar vi två microdialyskatetrar. I båda går det vätska med lite radioaktivt märkt adenosin. Men till en kateter så har vi även tillsatt ett ämne som blockerar aktiviteten hos enzymet PNP. Vi gör ischemisk prekonditionering vid varje försök, fyra korta perioder av strypt blodflöde följda av påsläppt flöde, innan en lång ischemisk period. Vi ser att alla våra uppmätta värden följer mönster som förväntat vid ischemi/reperfusion, men i den kateter som har enzymblockerare så får vi ingen förändring av nivån på radioaktivt märkt laktat. Vi har tolkat detta så att via blockeringen så får vi ingen uppreglering av PNP enzymet, alltså sker ingen ökad metabolism av adenosin till laktat.

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